

**THE RELATIONSHIP BETWEEN CHROMOSOME STRUCTURE AND
FUNCTION IN THE EUKARYOTIC GENOME
– HIGH RESOLUTION STUDIES USING SURFACE-SPREAD
POLYTENE (SSP) CHROMOSOMES**

DISSERTATION

zur Erlangung des Grades
eines Doktors der Naturwissenschaften
der Fakultät für Biologie
an der Ruhr-Universität Bochum, F.R.G.

vorgelegt von

Theodore E. Whitmore

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Referent: Professor Dr. W.-E. Kalisch

Korreferent: Professor Dr. A. Ruthmann

Dedicated to my best supporters-
Kornelia, Christopher, and Analena-Catherin

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ABBREVIATIONS

Chemicals, solutions, and buffers

BSA	albumin bovine
Chrome alum	potassium chromium sulphate
FITC	fluorescein isothiocyanate
Giemsa	azur eosin methylene-blue
Hoechst 33258	bisbenzimidazole H 33258
Nipagin	methyl 4-hydroxybenzoate
PBS	phosphate-buffered saline
SSC	standard saline citrate
Titriplex III	Na ₂ EDTA, ethylenediaminetetraacetic acid, disodium dihydrate
Tris	Tris (hydroxymethyl) aminomethane

Generic

<u>Ch. th. pi.</u>	<u>Chironomus thummi piger</u>
<u>Ch. th. th.</u>	<u>Chironomus thummi thummi</u>
<u>D. melanogaster</u>	<u>Drosophila melanogaster</u>
<u>D. hydei</u>	<u>Drosophila hydei</u>

Microscopy, Cytology, and Methodology

Å	ångstrom, one ten-billionth (10^{-10}) of a meter
DIC	differential interference contrast
EM	electron microscopy
kV	kilovolt
LSM	Laser Scan Microscope
LM	light microscopy
nm	nanometer, one-billionth (10^{-9}) of a meter
NC	nurse cell
OD	optical density
SEM	scanning electron microscopy
SSP	surface-spread polytene (chromosome preparation technique)



TC	trichogen cell
TEM	transmission electron microscopy
μm	micron, one-millionth (10^{-6}) of a meter

Nucleic acids, Antibodies, and Enzymes

biotin-11-dUTP	dTTP analog with biotin attached at the 5-position of the pyrimidine base by an 11-atom linker
dATP	2'-Deoxyadenosine 5'-triphosphate
dCTP	2'-Deoxycytidine 5'-triphosphate
dGTP	2'-Deoxyguanosine 5'-triphosphate
DNP	deoxyribonucleoprotein
EM GAR G20	immunoglobulin-gold conjugate suited for electron microscopy (EM). The letters GAR indicate the type of immunoglobulin used, in this case affinity-purified goat anti-rabbit IgG. The G followed by a number designates the mean diameter of the gold particles in nanometer, here G20 indicates, for example, colloidal gold with a 20 nm diameter.
FUdR	5-fluoruracil-2-deoxyribosid
IgG	immunoglobulin G
kb	kilobase
RNase A	ribonuclease A from bovine pancreas
RNase T1	ribonuclease T1 from <u>Aspergillus oryzae</u>

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PUBLICATIONS

In addition to unpublished material, parts of this dissertation are based on and have already appeared in the following original papers and abstracts (alphabetically listed). These will be referred to as I - XVI in the text.

- I Kalisch, W.-E., Schwitalla, G. and Whitmore, T. (1986a). Electron microscopic band-interband pattern of the X chromosome in Drosophila hydei. Chromosoma 93, 387 - 392.
- II Kalisch, W.-E., Schwitalla, G. and Whitmore, T. (1986b). Computerized EM maps of surface-spread polytene chromosomes II. Chromosome 4 in Drosophila hydei. Cytobios 45, 185 - 194.
- III Kalisch, W.-E. and Whitmore, T. (1983). Differences in the number of polytene chromosome bands as studied by electron microscopy. Cytobios 37, 37 - 43.
- IV Kalisch, W.-E. and Whitmore, T. (1986). The SSP chromosome preparation technique as to be used for Drosophila melanogaster. Drosophila Information Service 63, 142 - 146.
- V Kalisch, W.-E., Whitmore, T. and Reiling, H. (1984). Computerized EM maps of surface-spread polytene chromosomes. I. Digitizing and plotting of banding patterns. Cytobios 41, 47 - 62.
- VI Kalisch, W.-E., Whitmore, T. and Schwitalla, G. (1985a). Electron microscopic map of surface-spread polytene chromosomes in Drosophila hydei. Chromosoma 92, 265 - 272.
- VII Kalisch, W.-E., Whitmore, T. and Schwitalla, G. (1985b). Ultrastructure of polytene chromosomes in Drosophila hydei. Abstract. 9th European Drosophila Research Conference. Balatonszeplak, Hungary.

- VIII Kalisch, W.-E., Whitmore, T. and Siegel, A. (1985c). Laser scanning microscopy of surface-spread polytene chromosomes. *Journal of Microscopy* 137, 217 - 224.
- IX Reiling, H., Kalisch, W.-E. and Whitmore, T. (1984). Computerized EM chromosome maps. *Drosophila Information Service* 60, 172 - 174.
- X Reiling, H., Kalisch, W.-E., Whitmore, T. and Tegtmeyer, K. (1984). Computer map of salivary gland chromosomes in *Drosophila hydei*. *European Journal of Cell Biology* 34, 336 - 338.
- XI Whitmore, T. (1986). Localization of a "house-keeping" gene by *Chironomus thummi thummi*. *Cytobios* 46, 193 -200.
- XII Whitmore, T. and Kalisch, W.-E. (1984). Hoechst 33258 staining of surface-spread polytene chromosomes in *Drosophila hydei*. *Drosophila Information Service* 60, 206 - 207.
- XIII Whitmore, T. and Kalisch, W.-E. (1986). Using SSP chromosomes in fluorescence microscopy. *Stain Technology* 61, No.2, 79 - 81.
- XIV Whitmore, T., Kalisch, W.-E. and Reiling, H. (1984). An EM map of chromosome-6 of *Drosophila hydei*. *Drosophila Information Service* 60, 208.
- XV Whitmore, T., Kalisch, W.-E. and Schwitalla, G. (1983). EM photo maps in *Drosophila*. Abstract. 8th European Drosophila Research Conference. Edited by M. Ashburner. Cambridge, England.
- XVI Whitmore, T., Schwitalla, G. and Kalisch, W.-E. (1985). Incident light microscopy of SSP chromosomes. *Drosophila Information Service* 61, 201 - 203.

1. INTRODUCTION

Research committed to the investigation of the relationship between the structure and function of eukaryotic chromosomes is deeply enrooted in almost countless studies involving the giant polytene chromosomes commonly found in a number of larval, pupal, and adult tissues of dipterous flies and midges (for reviews see Beermann, 1972; Zhimulev et al., 1981; Richards, 1985). In-depth cyto- and molecular-genetic research on polytene chromosomes got its "start off the ground", so-to-say, after the fundamental discoveries that not only did these chromosomes seem to represent greatly amplified interphase chromosomes (Painter, 1933; Heitz and Bauer, 1933; Koltzoff, 1934; King and Beams, 1934), but that there also existed a colinear relationship between the genetic and chromosome maps (Bridges, 1937). Since then, cytologists and geneticists alike have sought to answer the question initially put forth by Painter (1934), who asked, "Where are the genes? Are they represented by the deeply staining material or by some other part of that region of the chromosome?" During the some 50 years which have followed, polytene chromosomes of the Diptera have been the subject of intense and, it must be noted, often contradictory cytogenetic studies and mutually opposing views (reviewed in Ashburner, 1980; Korge, 1987). They have served, nevertheless, as a convenient source of material for examination and have aided, thereby, in the envisioning of a number of different, but quite plausible models for the relationship between chromosome structure and function in the eukaryotic genome.

The polytene chromosomes characteristic pattern of bands (chromomeres) and interbands (interchromomeres) has led some to favor as locus for the genes just the bands (Bridges, 1935) or interbands (Koltzoff, 1934), some, both together (Crick, 1971; Paul, 1972; Sorsa, 1976; Rykowski et al., 1988) and others, each individually (Kosswig and Sengün; Speiser, 1974; Zhimulev et al., 1981). To elucidate

further on just four of these models- Crick (1971) postulated, for example, that "The coding sequences of the DNA (that is, all those sequences which code for polypeptide chains) are mainly, if not entirely, in the interbands...The bands, which contain all but a few percent of the DNA, are identified as control elements." Paul (1972) proposed, to the contrary, "that the interband regions are essentially polymerase-binding sites, and that transcription moves into the band regions from initiation sites which are likely to be situated near the band-interband junctions." Speiser (1974) hypothesized "that the interchromomeres are obligatory miniature-puffs, which are formed by the genes of "basic synthesis" (the synthesis which guarantees the functional cellular life)...The chromomeres...contain the loci of stage- and tissue-specific differential activity." And then Rykowski et al. (1988) enunciated the following model, based on their work with the Notch gene by Drosophila melanogaster, "that the regulatory region of a gene, even a quiescent one...is maintained in an open chromatin conformation, ready to accept the transcription factors and polymerase required for activation. The transcribed portion of the gene is stored in the band, most likely as 30 nm fibers, until activation."

Since the beginning of light microscopic (LM) analysis of polytene chromosomes, the squash preparation technique has been used for routine studies concerning the band-interband question. Here, chromosomes are usually fixed in a mixture of alcohol and acetic acid and separated from the other cellular material by squashing in just plain acetic acid. The bands and interbands observable light microscopically in well-extended chromosomes are then taken as being the structural units of the chromosomes. This technique has, however, several disadvantages:

- 1) Only in well-extended chromosomes sections can individual bands and interbands be identified light

microscopically, because of the juxtaposition of polytene structures.

2) Cytological depictions of the band-interband pattern in routine light micrographs show only part of the total number of polytene structures which are registered in detailed studies.

3) Finally, many smaller polytene structures (e.g. so-called "minibands") are at or beyond the limits of light microscopic resolution. Inadequate resolution can complicate the interpretation of results, for example, whether the labeling in immunofluorescence or autoradiographic studies comes from smaller bands or from the interbands.

It has also led to discordance in the conclusive answering of the several basic questions of primary interest in regards to the location of the genes such as 1) the total number of bands (interbands) in a genome, 2) the constancy of banding patterns in different tissues, and 3) the DNA content of the bands and interbands. Furthermore, due to the methodological and structural reasons mentioned above, there is a large discrepancy between the structural details which one can observe microscopically after analyses of well extended chromosomes from squash preparations and that of the photographic depiction of these patterns in individual routine chromosome preparations. In order to depict all of the structural details it is, therefore, necessary to construct so-called "chromosome maps". These are hand-drawn depictions of the band-interband pattern made after detailed analyses of large numbers of chromosome preparations.

A breakthrough in the pattern analysis of polytene chromosomes has been the squash and thin sectioning method with electron microscopic (EM) analysis (M. Sorsa and V. Sorsa, 1967), by which additional bands and hidden interbands

(compared with LM analyses) became visible and depictable. Although this technique has been used successfully so far for numerous detailed pattern and developmental analyses of sections of salivary gland chromosomes in Drosophila (e.g. Sorsa, 1982a,b; Saura, 1983; Semeshin et al., 1985a,b; Saura et al., 1988) and for one short chromosome in Chironomus tentans (ten Tusscher and Derksen, 1982), it is, in general, too laborious and time-consuming for routine work.

In Drosophila melanogaster EM maps exist as the result of the work of the Sorsa group over the past two decades (reviewed by Saura, 1986). For other Drosophila and Chironomus species, comparable EM maps have not even existed for single chromosomes until the material to be presented in this dissertation. In Drosophila hydei, EM maps had been published, for example, for only the distal half of chromosome 2 and for several smaller sections (Berendes and Meyer, 1968; Berendes, 1972; Berendes et al., 1974; Grond and Derksen, 1983). Various methods of ultrathin sectioning of unsquashed salivary gland polytene chromosomes (e.g. Pervov et al., 1975; Hill and Watt, 1977) have also been applied for examining a few chromosome sections by Chironomus thummi (Kiknadze et al., 1976), Chironomus tentans (Sass, 1980), and Drosophila melanogaster (Mott et al., 1980; Mott and Hill, 1986).

The comparisons between the light- and electron-microscopic analyses of the banding patterns of polytene chromosomes have indicated that mapping on the light microscopic level is no longer precise enough to fulfill the needs of modern chromosome research in Diptera. EM investigations have often shown 30 - 75% increases in the number of recognizable bands over previous LM studies (e.g. Sorsa, 1979; ten Tusscher and Derksen, 1982; Grond and Derksen, 1983). This, along with the recent advances in the molecular mapping of dipteran DNA and the chromosomal localization of cloned sequences (e.g. Barnett et al., 1980; Scott et al., 1983; P. Spierer and A. Spierer, 1983; Bossy et al., 1984;

Antoine and Niessing, 1984; Haenlin et al., 1985; Hankeln et al., 1988), has raised the necessity of seeking a method which would enable on a routine basis not only the modernizing of LM reference maps by EM photo maps and EM chromosome maps, but also to localize precisely, for instance, a hybridized cloned nucleic acid sequence. Several different methods have thus been developed to take advantage of the higher resolution of electron microscopy for the detection of sites of RNA synthesis (Semeshin et al., 1979; Kress et al., 1985), the location of polymerase B (or II) (Sass and Bautz, 1982a,b) and a major chromatin antigen (Hill et al., 1986) as well as for gene mapping of rRNA genes (Manning et al., 1975; Wu and Davidson, 1981). Due to the inherent technical problems, however, none of the methods have found any further use than in the initial publications cited above.

A new methodological possibility which leads to an improved photographic depiction of the band-interband pattern was introduced with the surface-spread polytene (SSP) chromosome preparation technique (Kalisch and Hägele, 1981; Kalisch, 1982a; Kalisch and Whitmore, 1983). In this technique, polytene chromosomes are spread, after an acid pretreatment, on an aqueous-surface in the lateral and longitudinal direction. It has been demonstrated that the method is suitable for chromosome mapping of high resolution without thin sectioning using light microscopy (Kalisch, 1982a,b; Kalisch and Hägele, 1982; Torramilans and Juan, 1985), transmission EM (Kalisch, 1982b; Kalisch and Hägele, 1982; Kalisch and Whitmore, 1983; Whitmore et al., 1983; Kalisch and Böhm, 1985) and scanning EM (Kalisch and Jacob, 1983) as well as being suitable for analyzing the banding patterns of different tissues (Kalisch, 1982b; Kalisch and Hägele, 1982). It was shown, in addition, to be usable for autoradiography at the LM level (Kalisch and Hägele, 1982) and in situ hybridization at the EM level (Kress et al., 1985).

This thesis involves the use and further development of the SSP chromosome technique's potential to attack some of the basic problems touched upon in the preceding paragraphs which are encumbering the further study and clarification of the relationship between the structure and function of polytene chromosomes. This necessitated taking a comprehensive approach involving full scale EM investigations directed at attempting to answer the following questions:

- Is the surface-spread polytene (SSP) chromosome preparation technique applicable for practical electron microscopic (EM) mapping purposes of entire genomes?
- Just how accurate are LM determinations of band numbers?
- What are the theoretical coding potentials of the bands and interbands?
- Is there a true intertissue constancy of the banding pattern of dipteran polytene chromosomes and to what extent do tissue specific variations really occur?
- Are transcriptionally active genes limited to just the interbands or puffed regions, just the bands, or do they cover both types of chromatin structure?

2. MAJOR OBJECTIVES

The objectives realized in this thesis are:

Cytological

- Electron microscopic photo maps of salivary gland chromosomes for the entire genome of Drosophila hydei and a comparison with the light microscopic based maps of Berendes (1963).

- An electron microscopic fine analysis and computerized chromosome mapping of the salivary gland chromosomes X, 4, and 6 in Drosophila hydei with quantitative determination of individual interband lengths and band thicknesses for the entire chromosomes.

- A comparative electron microscopic photo map analysis between the salivary gland and Malpighian tubule chromosomes for the entire genome of Chironomus thummi piger determining the general characteristics and differences in the physical appearance of intercalary heterochromatin, puffing patterns, telomeric regions, and constancy of the banding patterns.

- The first light microscopic and electron microscopic (EM) localization of a low copy "house-keeping" gene by in situ hybridization.

Methodological

- Optimization of the surface-spread polytene (SSP) chromosome preparation technique for various Diptera.

- Development of a novel routine method for transferring in situ hybridized and immunogold labeled

SSP chromosomes onto grids for electron microscopy and its use to locate a repetitive DNA sequence (the "Cla-element") and a "housekeeping" gene (the haemoglobin IV gene) by Chironomus thummi.

- Development of a method for scanning electron microscopy of non-coated or osmium treated chromosomes.

- Improvement of the resolution in fluorescence microscopy of the band-interband pattern and other chromosomal characteristics through the use of SSP chromosomes.

- Improvement in the structural depiction of polytene chromosomes at the light microscopic level by the use of incident light, differential interference contrast microscopy and laser scanning microscopy with SSP chromosome preparations.

3. MATERIALS

The materials used can be obtained from the following sources as noted. A complete address index is given at the conclusion of the materials section.

3.1. Stocklist

All of the stocks used are kept and maintained at the Institut für Genetik, Ruhr-Universität Bochum, P.O. Box 102148, D-4630 Bochum 1

- Drosophila melanogaster (Berlin), wild-type strain
Drosophila hydei (Düsseldorf, 1982; Sao Paolo),
 wild-type strain
Chironomus thummi thummi (H1) (Ost-Friesland, 1973)
Chironomus thummi piger (E) (Ost-Friesland, 1978)

3.2. Chemicals

- | | |
|--|------------------|
| Acetic acid | - Baker |
| Agar agar | - Nordwald |
| Albumin bovine (BSA) | - Serva |
| n-Amyl acetate | - Merck |
| Azur eosin methylene-blue (Giesma) | - Merck |
| Bisbenzimidazole H 33258 (Hoechst 33258) | - Serva |
| Blue dextran | - Sigma |
| Brewer's yeast | - Cenovis |
| Calcium chloride | - Merck |
| Calcium phosphate | - Merck |
| Chloramphenicol | - Sigma |
| Chloroauric acid | - Aldrich |
| Citric acid-1-hydrate | - Riedel-de Haën |
| Cornmeal | - Schmidt |
| Dextran sulfate 500 | - Serva |
| Ethanol | - Riedel-de Haën |
| Ethyl ether | - Roth |
| Formamide | - Baker |
| Formvar | - Serva |
| Gelatin powder | - Merck |
| Glycerol | - Serva |
| Hydrochloric acid | - Baker |
| Isopropanol | - Roth |
| Magnesium chloride | - Merck |
| Magnesium sulfate | - Merck |
| Manganese (II) chloride-4-hydrate | - Riedel-de Haën |
| 2-Mercaptoethanol | - Merck |

Methyl 4-hydroxybenzoate (Nipagin)	- Merck
Nitrocellulose, 6%	- Balzers
p-Phenylenediamine	- Sigma
Polyethylene glycol 20,000 (PEG 20M)	- Roth
Potassium carbonate	- Baker
Potassium chloride	- Merck
Potassium chromium sulphate	- Riedel-de Haën
Propionic acid	- Merck
RBS 50	- Roth
Silicone solution	- Serva
Sodium bicarbonate	- Merck
Sodium chloride	- Riedel-de Haën
Sodium citrate-2-hydrate	- Riedel-de Haën
Sodium hydroxide	- Riedel-de Haën
Sodium phosphate	- Sigma
Sugar beet syrup	- Schmitz
Titriplex III	- Merck
Tris	- Serva
Tris-HCl	- Serva
Urea	- Merck, Roth

3.3. Enzymes

Deoxyribonuclease-1	- Sigma
DNA-polymerase I (Kornberg polymerase)	- Boehringer
Ribonuclease A from bovine pancreas	- Serva
Ribonuclease T1 from <i>Aspergillus oryzae</i>	- Sigma

3.4. Deoxynucleotide triphosphates, DNAs

Biotin-11-dUTP	- BRL
2'-Deoxyadenosine 5'-triphosphate	- Sigma
2'-Deoxycytidine 5'-triphosphate	- Sigma
2'-Deoxyguanosine 5'-triphosphate	- Sigma
DNA from herring sperm	- Serva

3.5. Antibodies, conjugates, and sera

EM GAR G20	- Bio Cell/Plano
EM GAR G40	- Janssen
Goat anti-rabbit IgG	
(whole molecule) FITC conjugate	- Sigma
IgG fraction rabbit anti-biotin	- Enzo
Serum, goat-lyophilized	- Sigma

3.6. Glass-, metal-, paper-, and plasticware

Cover slips- 18x18mm; 24x24mm; 24x48mm	- Langenbrinck; Menzel/Fleishhacker
Containers- plastic, 3.5cm $\emptyset$; 5.5cm $\emptyset$	- Nunc
Culture vials- for <u>Drosophila</u> larvae	- Greiner
EM grids- type G 200, copper	- Veco
Filter paper- 5.5cm $\emptyset$; 3x6.5cm	- Macherey-Nagel
Forceps- EREM Swiss 5SA; Dumont- Dumoxel 4 Biologie	- Balzers
Membrane filters- pore size 0.45 μ m	- Sartorius
Microcaps- Drummond 10 μ l disposable glass micropipettes	- Bachofer
Microscope slides- ca. 76x26mm	- Langenbrinck
Microtiter plates- 10 μ l wells, disposable plastic, 60 wells/plate	- Nunc
Microtubes- Eppendorf 3810- 1.5ml	- Eppendorf
Parafilm- 4in x 125ft roll	- American Can Company
Syringe- glass, 100 μ l Hamilton, type 171011	- Fleischhacker
Syringe filter holder- disposable plastic, pore size 0.2 μ m	- Sartorius
Syringes- 1ml insulin, disposable plastic with 0.9 x 40mm needle	- Fleischhacker

3.7. Apparatus

Centrifuges- micro: Eppendorf 5415 ultra: Sorvall OTD-65B	- Eppendorf - Sorvall
Computers- IBM PC XT with HP7475A plotter; Sharp PC-1500 with Sharp CE-150 plotter	- local dealerships
Halogen lamp- Schott KL 1500	- Fleischhacker
Hotplate magnetic stirrers- Cenco; Ikamag RET-65	- Cenco - Fleischhacker
Membrane filter- 250ml capacity, type SM 16510	- Sartorius
Micro focus finder- for precision enlarger focusing on image grain	- Paterson
Micrometer- NSK, Japan Micrometer	- local sources
Microscopes- dissection: Wild, 6-50x; Zeiss OPMI g-MD light: Zeiss student model with phase contrast, 10/0.22 Ph1 objective; Zeiss photo-microscope II with Zeiss III RS fluorescence attachment; Zeiss photo-microscope equipped for incident light, differential interference contrast	- Wild - Zeiss - Zeiss - N/A - N/A - Zeiss
TEM: Siemens Elmiskop 101	- Zeiss
SEM: Jeol JSM 35	- N/A
LSM: prototype	- Zeiss
Ovens- Heraeus T 5042 E, 37°C; Memmert Pv18u, 100-220°C	- Heraeus - Fleischhacker

pH meter- WTW microprocessor pH 196	- WTW/Fleischhacker
Pipetman- Gilson P20, P200, P5000	- Abimed
Shaker- Heidolph type DSG 304	- Fleischhacker
Ultra sonic cleaner- Branson B3	- Fleischhacker
Vortex mixer- Genie 2	- Bender & Hobein/ Fleischhacker
Water baths- Haake F 423/4291, 100°C; Köttermann 3045, 42-65°C	- Köttermann

3.8. Sources of reagents and equipment- address index

Abimed Karl-Landsteiner-Str. 1 Ludwigshafener Str. 26 D-4000 Düsseldorf 1	ENZO Biochem Inc. Ortho Diagnostic Systems GmbH Karl-Landsteiner-Str. 1 D-6903 Neckargemünd
Aldrich-Chemie GmbH D-7924 Steinheim	Eppendorf Gerätebau Netheler & Hinz GmbH P.O. Box 650670 D-2000 Hamburg 65
American Can Company Dixie/Marathon Greenwich, CT. 06830	Fleischhacker KG Binnerheide 33 P.O. Box 1249 D-5840 Schwerte/Ruhr
Bachofer Laborgeräte- Glasbläserei D-7410 Reutlingen	Gibco/BRL GmbH P.O. Box 1212 Dieselstraße 5A D-7514 Eggenstein
J. T. Baker Chemicals Deventer, Holland	C.A. Greiner u. Söhne Abt. Labor technik Wuppertalerstr. 342 D-5650 Solingen-Gräfrath
Balzers Union AG P.O. Box 75 FL-9496 Fürstentum Liechtenstein	Gebrüder Haake KG Dieselstraße 4 Karlsruhe-Durlach
Bender & Hobein AG Zurich, Switzerland	Riedel-de Haën AG Wunstorfer Str. 40 D-3016 Seelze 1
Bio Cell Research Lab. P.O. Box 78 Cardiff CF11XL	W. C. Heraeus GmbH P.O. Box 1553 D-6450 Hanau 1
Boehringer Mannheim GmbH Biochemica P.O. Box 310120 D-6800 Mannheim	Janssen Life Sciences Products Lammerdries 55 B-2430 Olen, Belgium
Cenco Instrumenten N.V. Breda, Holland	
Cenovis Hefe Reform GmbH D-7760 Radolfzell	

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Otto Nordwald

Laboratorium für Fermente

und Organsubstanzen

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London WC1, England

Plano W. Plannet GmbH

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D-3550 Marburg 7

Carl Roth GmbH & Co. KG

Schoemperlenstraße 1-5

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Sartorius GmbH

P.O. Box 3243

D-3400 Göttingen

Fa. M. Schmidt

Mühle 64

D-4010 Hilden

Josef Schmitz KG

Grafschafter Krautfabrik

Meckenheim, Bezirk Köln

Serva

Feinbiochemica GmbH & Co.

P.O. Box 105260

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Sigma Chemie GmbH

Grünwalder Weg 30

D-8024 Deisenhofen

Sorvall Instruments

Du Pont GmbH

Dieselstraße 18

D-6350 Bad Nauheim

Stock Veco B.V.

Karel v. Gelreweg 22

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Wild

Heerburg, Switzerland

WTW

D-8120 Weilheim i. OB

Carl Zeiss

D-7082 Oberkochen

4. METHODS

4.1. The surface-spread polytene (SSP) chromosome preparation technique

The surface-spread polytene (SSP) chromosome technique was introduced in 1981 by Kalisch and Hägele and later improved by Kalisch and Whitmore (1983). Detailed descriptions of the optimized methodology were given in Kalisch et al. (1984) for Chironomus salivary gland chromosomes and in Kalisch and Whitmore (1986) for Drosophila salivary gland chromosomes. The following is the complete general procedure which was used in this study for the preparation of salivary gland chromosomes by Drosophila and for salivary gland and Malpighian tubule chromosomes by Chironomus.

4.1.1. Preparation of salivary glands and Malpighian tubules (for subscripts see page 18)

DROSOPHILA¹- salivary glands: After briefly washing the larva in pre-treatment solution², the salivary glands were excised with forceps in ca. 16 μ l of pre-treatment solution. The glands were washed in 8 μ l of pre-treatment solution in a 10 μ l well of a disposable 60 well microtiter test plate. The glands were then transferred to a new 10 μ l well, also filled with 8 μ l of pre-treatment solution. Here, after removing any remaining parts of the fat bodies, the glands were mechanically lacerated with forceps. This procedure was observed using a dissecting microscope, where the bursting of the cells was visible. The lacerating of the glands was continued, if necessary, until the glands disappeared into a turbid "chromosome suspension". The pre-treatment procedure was terminated after 5 min (from the excising of the glands) by the spreading procedure.

CHIRONOMUS:- Malpighian tubules: After draining off excess water on the larva on a strip of filter paper, the Malpighian tubules were excised in ca. 16 μ l of pre-treatment solution. The tubules were immediately transferred to 6 μ l of pre-treatment solution in a 10 μ l well of a disposable 60 well microtiter test plate. Here, the tubules quickly burst releasing the chromosomes into the pre-treatment solution. There was usually no need for mechanical laceration. The empty tubules were then removed from the well. The incubation time was a maximal 3 - 5 min after the removal of the tubules. The pre-treatment process was terminated by the spreading procedure.

CHIRONOMUS- salivary glands: The excised glands were washed in 8 μ l of pre-treatment solution in a 10 μ l well of a disposable 60 well microtiter test plate. The glands were then immediately transferred into a fresh 8 μ l of pre-treatment solution and mechanically lacerated with forceps. After 10 min the glands were lacerated again and the salivary plugs removed. The suspension was incubated for another 30 min followed by the spreading procedure.

In cases where both the salivary glands and the Malpighian tubules were required from an individual larva, the more fragile Malpighian tubules were excised first and then the salivary glands.

4.1.2. Spreading procedure (Figure 1)

A semispherically shaped drop (ϕ = ca. 1.5 cm) was formed from 1.5 ml of spreading medium[®] on a double siliconized[®] microscope slide using a Gilson P5000 Pipetman. A siliconized[®] Microcap[®] was used to transfer the pre-treated chromosomes (the "chromosome suspension") from the 10 μ l well to the surface of the spreading medium. For each spreading procedure a ca. 4 μ l drop of the "chromosome suspension" was formed at the lower end of the Microcap and after waiting for about 30 sec the drop was brought into contact with

the zenith of the spreading medium surface. At the moment when both drops touched each other the semispherically shaped drop of the spreading medium was clearly flattened. In those cases where the spreading medium did not show this effect, the Microcap had either been pushed through the surface of the spreading medium or too small amount of "chromosome suspension" had been used. In either case the spreading procedure had to be started again using a new drop of spreading medium.

4.1.3. SSP chromosomes

The spread chromosomes⁶ remained on the surface of the spreading medium. They could be visualized using a phase contrast optic (Achromat 10/0.22 Ph1). In order to enlarge the area of visibility in connection with the curved surface of the spreading medium, the surface of the spreading medium was mechanically flattened⁷. Spread chromosomes slipping to the rim of the surface of the spreading medium could be brought back by blowing air⁸ over the surface of the spreading medium from different directions.

4.1.4. Picking up the chromosomes (Figure 2)

SSP chromosomes were picked up on carbon-coated Formvar grids⁹. In order to obtain the entire band-interband pattern of a chromosome or even an entire genome, grids were used having one central 500 x 1000 μm mesh. For the process of picking up a chromosome, a pair of forceps (holding the grid) were secured in a seesaw¹⁰ type construction. Through this arrangement, it was possible to visualize both the grid and the SSP chromosome simultaneously allowing the proper adjustment of the mesh over the chromosome.

After picking up a SSP chromosome by touching the spreading medium with the grid, the EM grid was carefully washed¹¹ by floating the grid, filmed side down, for 10 min on the surface of aqua bidist., dipping several times in

50% and transferring to 96% isopropanol for 10 min. Air-drying of the grids (filmed side up) was done on a filter paper (since the copper grids had been bent slightly before being covered with the Formvar film, a contact between the film and the filter paper could be avoided). There was no contrasting procedure used with this technique. The grids could be stored without any apparent changes in the chromosomal structures, but time appeared to weaken the strength of the Formvar film.

For LM analyses, SSP chromosomes were picked up on subbed slides¹² or cover slips (for this procedure, the subbed slide was held at an angle of 90° to that of the slide where the spreading medium was on). If the SSP chromosomes were blown from the rim to the middle of the surface of the spreading medium after the pick up, it was possible to get even a second and a third LM preparation from the same drop of spreading medium. The washing was the same as for the EM grids, except done with the slide standing vertically in a coplin jar.

4.1.5. Cytological analysis

A first analysis of the degree of spreading and the quality of the SSP chromosomes on the EM grids was done light microscopically (16/0.40 Ph 2 objective). For the EM analysis of the SSP chromosomes a Siemens Elmiscop 101 was used at 40 and 60 kV, respectively. Micrographs were usually taken with a 1,600x or a 2,800x magnification and used with a final magnification of 3,200x and 5,600x, respectively, unless overviews had to be given with a lower magnification.

Subscripts of sections 4.1.1. - 4.1.5.

1. Raising of the larvae:

DROSOPHILA larvae can be readily cultured on a wide variety of media. The one used for this study was prepared as following: for ca. 1,200 ml (sufficient for 60 small culture vials), 100 ml sugar beet syrup, 75 g cornmeal, 15 g agar and 18 g Brewer's yeast were mixed in 200 ml of cold water and added, while stirring, to 750 ml of boiling water and allowed to cook for 5 min by medium heat. Before pouring the medium, 6 ml of propionic acid and ca. 50 mg each of methyl 4-hydroxybenzoate (Nipagin) and chloramphenicol were added as fungicides. The culture conditions were the same as commonly used by others for yielding proper chromosome squash preparations. The second- and third-instar larvae were raised at 18°C and fed with a daily supplement of Baker's yeast. In order to ensure an optimal food supply, third-instar larvae were transferred once to fresh medium.

CHIRONOMUS larvae were cultured as described by Hägele (1975) at 15 - 21°C.

2. Pre-treatment solution: For this 3.18 M citric acid 1-hydrate and 8.82 M propionic acid solution, 10 g of a citric acid 1-hydrate was dissolved in aqua bidist. by stirring and gentle heating and brought to 15 ml. Separately, 19.8 ml of propionic acid was mixed with 10.2 ml aqua bidist. Both acid solutions were carefully mixed together and could be stored in a tightly stoppered vial for several days. Depending upon the conditions of the stock being studied, other ratios of the solutions than the 1:2 above (for example, 1 part propionic to 1 part citric acid) were necessary to provide the required degree of chromosome spreading.

Subscripts of sections 4.1.1. - 4.1.5. con't.

3. Spreading medium: The 4 M urea and 0.1 M HCl solution was prepared as following: 24 g urea and 10 ml HCl were brought to 100 ml aqua bidist. A pH of 2.0 was achieved by additional 1 M HCl.

4. Siliconizing: A commercially available solution (Serva) was used for siliconizing.

Microscope slides- After coating the microscope slides with silicone solution, they were dried horizontally for 60 min at 100°C. This prevented a gradient of silicone from being built up from one side to the other which would result in a movement of the spreading medium to one side when the drop was formed or during the spreading process. The slides were siliconized twice. Each slide was used for only one preparation.

Microcaps- 100 micropipettes were dropped into a beaker glass filled at the bottom with a small amount of silicone solution. Capillary action drew the silicone solution up into the micropipettes which were then removed from the beaker glass and the excess silicone solution shook out. The micropipettes were dried vertically for 60 min at 100°C.

5. Microcap: The 10 μ l Microcap was connected (using the Microcap holder) with a paraffin-oil filled 100 μ l Hamilton syringe. The plunger of the syringe was connected directly with a micrometer. An airspace of maximal 3 cm was left between the Microcap connection and the start of the paraffin-oil. The siliconized Microcap was discarded after every few spreading procedures.

Subscripts of sections 4.1.1. - 4.1.5. con't.

6. Spread chromosomes: Well-spread chromosomes were more or less free of those types of thick protein conglomerates which strongly reflect light as usually seen in squash preparations. Extremely well-spread chromosomes had a very low contrast by the optical system used making it difficult to locate them at first.

7. Flattening of the spreading medium: A disposable syringe was used which was connected with a rubber tube connector serving as a mouthpiece. The syringe needle (0.9 x 40 mm) could be injected at any area of the spreading medium. With the needle near the center (to avoid too much circulation) the spreading medium was sucked off very slowly to prevent a deformation of the drop at the rim. In those cases where inlets did appear, they could be removed by moving the syringe needle (together with the adherent spreading medium) to the old border of the rim.

8. Air blowing: A mouth controlled rubber tube was used which was capped with a disposable Pipetman C20TJ tip. The extent to which this technique worked depended upon the amount of cellular material accompanying the SSP chromosomes on the surface of the spreading medium. Furthermore, it should be emphasized, that a flattened surface of the spreading medium supported the efficiency of the procedure.

9. Formvar grids: The filming of grids was carried out with certain modifications as described by Lickfield (1979), whereby the humidity used played an important role in the preparation of optimal films: a) Several microscope slides were cleaned with a linen cloth which had been boiled beforehand in distilled water. This was repeated with a new cloth. b) A 0.25 - 0.35% solution of Formvar was prepared in chloroform (or in 1,2-dichlorethane) and stored in a dark bottle with an opening large enough for a

Subscripts of sections 4.1.1. - 4.1.5. con't.

microscope slide. c) By 38% humidity one of the clean microscope slides was dipped into the Formvar solution, pulled out slowly, and left to dry for ca. 24 hr at the same humidity. A plexiglass box was used for this step which had an opening to prevent a fogging of the film and a typical dry gel to keep the humidity of the film at ca. 38%. The opening in the plexiglass box was also kept relatively closed with a type of flexible (vinyl) plastic. d) The dried, film-coated slide was scored along the edges with a razor blade. e) The filmed microscope slide was now slowly slid into a petri-dish filled with distilled water at an angle of ca. 5 - 10°. Thereby, the film on the microscope slide floated onto the surface of the water. f) The defilmed microscope slide was pulled out of the petri-dish. g) Electron microscope grids were placed with the dull side down on the film. h) A strip of either filter paper or parafilm was lowered onto the grids and as soon as it stuck to the film, the strip was pulled with the grids out of the petri-dish. To prevent contact between the film and the filter paper the grids were bent slightly beforehand. i) The filter paper (Parafilm) strip was placed with the filmed EM grids on top in a petri-dish lined with filter paper to dry. j) The petri-dish was closed with a glass lid. k) In order to further stabilize the film the grids were often carbon coated.

10. Seesaw: A forceps frame was used consisting basically of two parts (1 and 2 in Figure 2) which served the function of a pivot point for the forceps. The forceps could be swivelled with the one end holding the grid and the other one being used for hand-directioning. Part 1 of the seesaw (a 25 x 20 x 15 mm aluminum block with a 4 x 10 x 15 mm lip on which the part 2 was attached with a screw) could be moved on the microscope table. Part 2 was an "instrument

Subscripts of sections 4.1.1. - 4.1.5. con't.

holder" (part of the micromanipulator "de Fonbrune", type PM1; Bachofer GmbH).

11. Washing procedure: The Formvar film was not very strong when it was wet. For the wash in 50% isopropanol the grids were only moved with the film in the vertical direction. Plastic containers (35mmØ x 10mm) were used for these processes and disposed of after half a day to avoid protein contamination.

12. Subbed slides: Prepared basically as described by Atherton and Gall (1972). For this, 5g of gelatin was dissolved in 1000 ml of distilled water at 30 - 35°C to which 0.5 g potassium chromium sulphate (chrome alum) was given. After cooling, the solution was filtered. Slides were dipped into the solution, dried vertically and then allowed to cure overnight at 30°C.

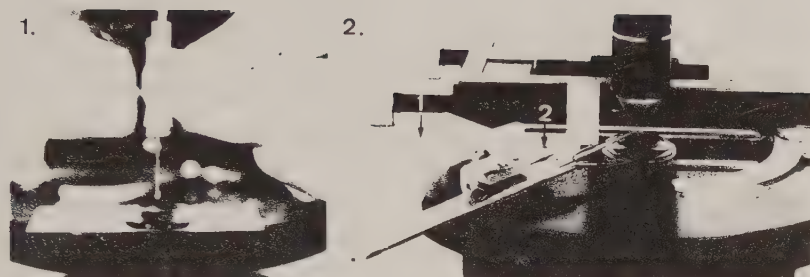


Figure 1. An illustration of the spreading procedure used in the surface-spread polytene (SSP) chromosome preparation technique. The "chromosome suspension" is hanging as a small drop at the lower end of the Microcap. This is immediately before the contact with the spreading medium (large drop). After the spreading process, the microscope slide is moved using the specimen holder into the phase optic. (Source: IV)

Figure 2. An illustration of the device used to pick up SSP chromosomes on EM grids. The seesaw holding the forceps with the grid consists of two parts (indicated by arrows 1 and 2) and can be moved freely on the microscope table to bring the mesh of the grid into alignment with the chromosomes (both of which can be seen with the phase optic). The actual distance is shown between the objects and the objective being used. See also subscript 10 for more details. (Source: IV)

4.2. Computerized electron microscopic (EM) maps of SSP chromosomes

For digitizing and plotting of banding patterns, a standard plotting program was written in Extended Basic originally for a pocket computer (Sharp PC-1500) coupled to a plotter (Sharp CE-150) with a maximum speed of 11 characters/sec (Kalisch et al., 1984; Reiling et al., 1984). In this program, the individual polytene structures (i.e. one chromosome band and its right-handed interband) was digitized using a set of variables: (A) band diameter, (B) band type—solid or dotted, straight or curved bands, (C) band thickness, (D) interband length, (E) interband as well as puff and Balbiani ring outlines, (R) radius— for curved bands, (W) sectioning— of the pattern in chromosome maps and (V\$) reference numbers. The RAM capacity of the PC-1500 was extended to ca. 11.5 Kbytes with a memory module (Sharp CE-155).

The CE-150 had an X- and Y-axes plotter system with four printing directions and a minimum pen shift width of 0.2 mm. The plotter used a printing paper stripe with a 58 mm width, and 44 mm of this could be used for plotting. A minimum of 5.6 mm was used for chromosome sectioning, 32 (160 x 0.2 mm) as maximum band or puff diameter for the chromosome and 5.4 mm for the linear scale.

The PC-1500 did not have a real computer graphic, but a graphic mode with eight line types (i.e. with different lengths of lines and spacers). This was used to characterize the differences in the prominence of the chromosome bands (band type: variable B in the plotting). The computerized plotting of the chromosome maps was based on an undrawn center line from which the bilateral polytene structures of the chromosomes were constructed. Data which was intended to be used for more than a single plotting was

stored on standard tape cassettes (90 min) on a recorder (Sharp CE-152).

The program was later adapted for use with an IBM PC XT (MS-DOS; Basic) and a HP7475A plotter. The plotting program was similar to the one already published, but in the latter program automatic plotting of the chromosome outlines had been additionally integrated. Minor changes had to be made in accordance with the differences in both Basic versions used and the specific graphic language (HP-GL) of the plotter.

The HP7475 plotter had a pen diameter of 0.3 mm with a minimum pen shift width of 0.025 mm on a 297 x 420 mm area. The original plots had a 1:1 relationship with the polytene structures in the electron micrographs. For these plots, band thicknesses and interband lengths had to be grouped into 0.3 mm classes, according to the diameter of the plotter pen used and to enable diminished presentations of the plots. Through this, a better correlation could be achieved between the cytological details and the map as compared with the simpler equipment first used.

4.3. In situ hybridization of SSP chromosomes at the electron microscope level with biotin-labeled DNA and immunoglobulin gold conjugate markers

4.3.1. Preparation of colloidal gold (gold sol)

Monodisperse gold suspensions (colloidal gold) of 20 - 25 nm and 40 - 45 nm particle size were prepared basically according to Frens (1973) by the reduction of chloroauric acid with sodium citrate in aqueous solution. It should be emphasized that all of the glassware had to be very clean

and that all solutions were microfiltered (0.22 μ m). It was also best to prepare all of the solutions on the same day.

For 20 - 25 nm colloidal gold, 35 ml aqua bidist. was brought to boiling, then 350 μ l 1% HAuCl_4 was added followed by 600 μ l 1% $\text{Na}_2\text{-citrate}$ under vigorous stirring (with a magnet stirrer). The solution turned blue-black after about 25 - 30 sec and after about 70 sec orange-red. If the color was dark red or violet then the spheres were larger than 25 nm. The solution was refluxed for 15 min and allowed to cool down to room temperature while stirring. For 40 - 45 nm colloidal gold, the procedure was identical, but 450 μ l 1% $\text{Na}_2\text{-citrate}$ was used and the solution was refluxed for 30 min. The solutions were stored at 4°C and used within 2 weeks.

4.3.2. Adsorbing goat anti-rabbit antibodies to colloidal gold

Affinity-purified goat anti-rabbit immunoglobulin was absorbed to the colloidal gold as described by Kress et al. (1985) and Kress (personal communication).

Here, ca. 3 drops of 1% PEG 20 M were added to 14 ml of gold sol and then the pH adjusted to a value of 7.6 by adding ca. 70 - 75 μ l of 0.2 M K_2CO_3 . The salt flocculation test (Geoghegan and Ackerman, 1977) was used to determine the lowest concentration needed of the IgG to stabilize the gold sol. For this, 300 μ l gold sol probes were mixed with 10 μ l of PBS¹ containing increasing concentrations of IgG (from ca. 1 μ g/ml - 10 μ g/ml). The optical density (OD) was measured at 430, 525, and 700 nm and then 53 μ l of 10% NaCl was added. After 10 min the OD was measured again at 430, 525, and 700 nm.

Stabilized solutions followed the same form of the curves made from the probes before the addition of the NaCl. The control or unstable probe showed a sharp increase in the OD

reading between 525 and 700 nm. The amount of IgG needed for stabilization was added to the remainder of the respective pH-adjusted gold sol (usually between 1 - 5 $\mu\text{g/ml}$). After 10 min, 200 μl of a 3.5% PEG 20 M solution was added as a stabilizing agent.

Removal of excess or loosely bound antibodies was done by washing through centrifugation. The IgG gold conjugate was pelleted by centrifugation by 14,000 g for 1 hr at 4°C. The pellet was resuspended in Hutchison's buffer B² (Hutchison et al., 1982) and centrifuged again using the same conditions. The pellet was resuspended in 5 ml Hutchison's buffer B and the OD₅₂₅ determined (10 μl in 340 μl PBS). The stock solutions were stored at 4°C. Commercially produced immunogold reagents were also used.

Subscripts of section 4.3.2.

¹PBS (phosphate-buffered saline) ²Hutchison's buffer B

per liter:	10	g	NaCl	0.05	M	Tris (pH 7.0)
	0.25	g	KCl	0.15	M	NaCl
	1.44	g	Na ₂ HPO ₄	1	mM	MnCl ₂
	0.25	g	KH ₂ PO ₄	0.5	mg/ml	PEG 20 M

4.3.3. Biotinylation of cloned DNA by nick translation

The nick translation reaction is based on the enzymatic activities of pancreatic DNase I and *E. coli* DNA polymerase I. In brief, DNase I puts nicks into DNA molecules whereas polymerase I serves the dual purpose of being both an exonuclease and a polymerase which starts at the nicked sites removing the nucleotides in the 5'-3' direction and replacing the unlabeled nucleotides with either radioactively- (Rigby et al., 1977) or biotin-labeled nucleotides (Langer et al., 1981) with the 3'-hydroxyl group of

the terminal nucleotide acting as primer (Kelly et al., 1970).

The following was a modification of an Enzo Bio System protocol (Enzo Biochem, Inc, New York) for the incorporation of biotin-11-dUTP (a dTTP analog) into DNA.

In an 1.5 ml Eppendorf microtube standing in ice the following components were given in the order listed:

5 μ l	nick buffer stock solution ¹
1 μ l	2 mM dATP
1 μ l	2 mM dGTP
1 μ l	2 mM dCTP
5 μ l	0.4 mM Bio-11-dUTP
x μ l	DNA (= 1 μ g)
y μ l	distilled H ₂ O

Where "x" is the quantity needed to equal 1 μ g of DNA probe and "y" the quantity needed to yield a final volume of 50 μ l.

The refrigerated DNase I stock solution² was now diluted 10⁴-fold with ice-cold DNase buffer³. 6 μ l of the DNase I dilution was then added to the mixture above followed by 2.4 μ l polymerase I (= 12 units). The reaction mixture was incubated for 2 hr at 14°C and the reaction stopped by adding 5 μ l stop buffer⁴ and heating to 65°C for 10 min. 6 μ l was then removed for electrophoresis and, in cases where no radioisotope tracer (³H-dATP) was used, the labeled DNA was mixed with 12.5 μ l 0.5% blue dextran (BRL Focus 7(1):8-Bethesda Research Laboratories, Gaithersburg, MD; Pliley et al., 1986).

The labeled DNA was separated from unincorporated nucleotides by ethanol precipitation. Here, 50 μ l of sonicated denatured herring sperm DNA (1 mg/ml) was added as carrier followed by 200 μ l chilled 100% EtOH. This was stored over-

night at -20°C and then centrifuged for 15 min at 10,000 rpm by 4°C . The supernatant was discarded and the pellet washed with 1 ml 70% EtOH followed by a 5 min centrifugation as above. The supernatant was discarded and the pellet dried in a vacuum desiccator. The pellet was then dissolved in 100 μl TE buffer³ and the labeled DNA stored at -20°C .

Subscripts of section 4.3.3.

¹Nick translation buffer stock solution

0.5 M Tris-HCl (pH 7.5)

0.1 M MgCl_2

²DNase stock solution

1 mg/ml DNase I in 0.1 M MgCl_2 ;

stored at -20°C

³DNase buffer

10 mM Tris-HCl (pH 7.5)

1 mg/ml BSA

⁴Stop buffer

300 mM EDTA (pH 8)

⁵TE buffer (pH 7.5)

10 M Tris-HCl

0.1 mM EDTA

4.3.4. In situ hybridization of SSP chromosomes with biotinylated DNA and subsequent labeling with IgG gold conjugates

When biotinylated DNA probes are hybridized to chromosomes each biotin molecule can serve as an antigen to bind a primary antibody (for example, a rabbit anti-biotin antibody). The hybridization sites can then be labeled and thus visualized with a secondary antibody (for example, a goat anti-rabbit IgG gold conjugate) (Langer-Safer et al., 1982; Hutchison et al., 1982). The procedure described here was a modification of a protocol worked out especially for surface-spread polytene chromosomes by Kress et al. (1985).

SSP chromosome preparations: SSP chromosomes were prepared as described in section 4.1., but were picked up on nitrocellulose coated cover slips¹ which were affixed beforehand to normal microscope slides for easy handling.

RNase digestion: The chromosome preparations were treated with a mixture of RNase A (50 µg/ml) and RNase T1 (40 µg/ml) in 0.1 x SSC (sodium chloride/sodium citrate)² for 1 hr at 37°C in a sealed moist chamber. The slides were then rinsed in 0.1 x SSC, ethanol dehydrated, and air dried.

Chromosome denaturation: The chromosomal DNA was denatured for 2 sec in boiling 0.1 x SSC, placed in chilled 0.1 x SSC on ice, ethanol dehydrated, and air dried as above.

Hybridization conditions: Biotin-labeled DNA probes were heat-denatured (3 - 5 min in boiling H₂O) and hybridized to SSP chromosomes using the following two protocols: 1) in a solution of 50% formamide (deionized and recrystallized), 10% dextran sulphate, and 4 x SSC, overnight at 42°C in a sealed

moist chamber. Slides were washed three times in 2 x SSC and three times in PBS (phosphate-buffered saline)² to remove the unhybridized probe. 2) In a solution of either 5 x SSC or 3 x SSC, overnight at 55 - 60°C in a sealed moist chamber. Unhybridized probe was removed as above.

Detection of hybridized probes: All preparations were incubated for 6 hr with 20 - 25 μ l of a 1:25 μ l dilution of rabbit anti-biotin IgG in PBS containing 2 mg/ml BSA in a moist chamber at 37°C, then rinsed twice for 20 min in PBS and further incubated for 1 hr at 37°C with 20 - 25 μ l of a 1:10 dilution of normal goat serum. During this incubation time, the stock solution of goat anti-rabbit IgG gold conjugate was centrifuged for 20 min at 2,000 rpm to remove conglomerates and then an appropriate working dilution was made in Hutchison's buffer B. By IgG gold conjugates of large particle size (e.g. $\geq$ 40 nm), the number of conglomerates can be reduced by also centrifugating the working dilution as described above for the stock solution. The preparations were now rinsed twice for 15 min in PBS and incubated with 25 μ l of IgG gold conjugate per cover slip for ca. 4 - 16 hr. The preparations were then washed twice for 30 min in PBS and stained for 20 min with a 10% Giemsa solution⁴. The preparations were rinsed twice in phosphate buffer⁵, once in H₂O, once in 50% isopropanol, and air dried.

Subscripts of section 4.3.4.

¹Nitrocellulose coated cover slips: 24 x 48 mm cover slips were used either as supplied by the manufacturer or cleaned by soaking for 20 min in 10% RBS-detergent solution, rinsing in demineralized water, and aqua bidist., followed

Subscripts of section 4.3.4. con't.

by rinsing in 96% isopropanol, and air drying. The cover slips were affixed to pre-cleaned 76 x 26 mm microscope slides at the upper end with rubber cement. After the cement had dried the slides were dipped into a freshly prepared BSA (albumin bovine) solution (0.4 mg/ml). The BSA subbed slides were allowed to dry overnight and then coated with a 0.7% nitrocellulose solution in amyl acetate and allowed to dry vertically.

²SSC (standard saline citrate)

150 mM NaCl

15 mM Na₂-citrate

³PBS (phosphate-buffered saline)

see section 4.3.2., subscript 1

⁴Giemsa solution, 10%

10 ml concentrated Giemsa

90 ml 25 mM Phosphate buffer (pH 7)

⁵Phosphate buffer (pH 7)

Add 25 mM KH₂PO₄ to 25 mM Na₂HPO₄ until a pH of 7 is obtained.

4.3.5. Transferal of immunogold labeled SSP chromosomes onto grids for electron microscopy

Although the following procedure is described for a single chromosome, routinely several chromosomes could be removed from one cover slip. The immunogold labeled SSP chromosomes were on BSA-subbed and nitrocellulose coated cover slips

affixed to normal microscope slides as described in section 4.3.4., subscript 1.

The chromosome intended to be transferred was located using a microscope with phase optic (Achromat 10/0.22 Ph 1) and spotted with 5 μ l of a 1% nitrocellulose in amyl acetate solution. The slide was held semivertically and the excess liquid drained off using the corner of a 3 x 6.5 cm strip of filter paper. The nitrocellulose was allowed to dry with the slide standing vertically in a microscope slide rack. The chromosome was sealed through this, so-to-say, in a protective nitrocellulose sandwich.

An EM grid was then placed over the chromosome using a fine EM forceps and the grid encircled using a diamond tipped glass cutter. The EM grid was removed and the preparation air blown free of any glass particles which may have encured from the encircling process. The slide was now slowly lowered into a bowl of aqua bidist. at an angle of ca. 5 - 10°. With a fine EM forceps, the encircled area containing the chromosome could be pulled off the cover slip and onto the surface of the aqua bidist. Before the encircled area was completely removed from the cover slip, the slide was removed and placed again under the phase contrast microscope where an EM grid was placed exactly over the chromosome using a fine EM forceps. The preparation was then lowered once again into the aqua bidist. and the chromosome preparation with the grid floated completely off of the cover slip. Prevented in this manner, was any slippage of the grid from its position over the chromosome.

The grid was picked up from the aqua bidist. using one of two methods: a) on a strip of filter paper as described in section 4.1., subscript 9(8) or b) by lifting the first grid and the filmed chromosome out with another grid held underneath the first one with a fine EM forceps. The upper grid was steadied during the process with an EM forceps allowing the proper alignment of the both grids. The two

grids with the filmed chromosome between them were placed on filter paper and the upper grid removed. The film remained on the lower grid. The latter method proved to work most reliably. In both cases, the filmed grid was dried on filter paper in a plastic disposable container (5.5 cm Ø) until viewed with the electron microscope.

4.4. In situ hybridization of polytene chromosome squash preparations with biotinylated DNA and subsequent immunological labeling with FITC-conjugated antibodies

Squash preparations of polytene chromosomes were routinely used for preliminary and comparative purposes. At the light microscope level, in situ hybridized biotinylated DNA probes can be visualized using, for example, immunoglobulins coupled with fluorescein isothiocyanate (FITC). The protocol below was based on the one described by Langer-Safer et al. (1982) as modified by Whitmore (1986).

Standard squash preparations were prepared of salivary gland chromosomes from fourth instar larvae of Chironomus stocks by squashing in 50% acetic acid using subbed slides and siliconized cover slips, freezing (cover slip down) on dry ice and after flipping the cover slip off with a razor blade, immediate plunging of the preparation into 96% isopropanol. Squash preparations of salivary gland chromosomes from late third instar Drosophila larvae were made as described by Atherton and Gall (1972). Preparations were stored either air dried or at -20°C in absolute isopropanol.

Chromosome preparations were RNase digested, denatured, and hybridized as described in section 4.3.4. with the exception that the chromosomal DNA was denatured for 3 - 5 sec in boiling 0.1 x SSC instead of for 2 sec.

The hybridized probe was detected as described by Langer-Safer et al. (1982) with slight modifications. All preparations were incubated for 6 hr with 20 - 25 μ l of a 1:25 dilution of rabbit anti-biotin IgG in PBS containing 2 mg/ml BSA in a moist chamber at 37°C, then rinsed twice for 20 min in PBS¹ and further incubated for 1 hr at 37°C with 20 - 25 μ l of affinity isolated goat anti-rabbit IgG FITC conjugate diluted 1:50 in PBS containing 2 mg/ml BSA. The slides were rinsed twice in PBS for 15 min and mounted in anti-fading mountant².

Slides were examined and chromosomes photographed using a Zeiss photo microscope with a Zeiss III RS fluorescence attachment on Ilford HP 5 film exposed at 800 ASA. The slides were then rinsed, Giemsa stained as described by Pardue and Gall (1975), and the chromosomes photographed with phase contrast using Agfapan film at 25 ASA.

Subscripts of section 4.4.

¹PBS (phosphate-buffered saline)

see section 4.3.2., subscript 1

²Anti-fading mountant for fluorescence microscopy- Modified after Johnson and Araujo (1981)

Add 10 ml of Tris-buffered saline [0.05 M Tris (pH 7.8)/0.015 M NaCl] containing 100 mg of p-phenylenediamine to 90 ml of glycerol. Store in the dark at -20°C.

4.5. Hoechst 33258 staining of surface-spread polytene chromosomes

SSP chromosome preparations were stained with the DNA-specific fluorochrome Hoechst 33258 (Weisblum and Haenssler,

1974) as described for Drosophila hydei in Whitmore and Kalisch (1984) and for Chironomus thummi piger in Whitmore and Kalisch (1986).

For this, SSP chromosome preparations were made and picked up on subbed slides as described in section 4.1. The chromosomes were stained for 10 min with Hoechst 33258 [2 mg/ml in McIlvaine buffer¹ (pH 4.0)]. The chromosomes were then rinsed in distilled water, mounted in the same McIlvaine buffer, cover-slipped, sealed with rubber cement, and stored in the dark for 48 hr. Photographs were taken using a Zeiss photo microscope with a Zeiss III RS fluorescence attachment on Kodak Tri-X film at 400 ASA.

Subscript of section 4.5.

¹McIlvaine buffer (pH 4.0) - As described by Ruthmann (1970)

Add 61.4 ml 100 mM citric acid-1-hydrate to 38.6 ml 200 mM Na₂HPO₄

4.6. Scanning electron microscopy (SEM) of SSP chromosomes without conductive staining

A SEM technique was developed enabling high resolution viewing of SSP chromosomes without prior metal coating or osmium impregnation. The chromosomes are thus still fully suitable for immunological studies.

The following procedure was used: 24 x 48 mm cover slips were cleaned as described in section 4.3.4. subscript 1 and sputter coated with a 8 nm layer of gold. The gold covered cover slips were heat-tempered at 220°C for 60 min. This

helped to prevent the gold layer from flaking off in any of the solutions used later in the SSP chromosome preparation technique. The cover slips were then attached for easy handling to pre-cleaned 76 x 26 mm microscope slides at the upper end with rubber cement. SSP chromosome preparations were picked up on the slides as described in section 4.1.4. and checked for suitable chromosomes using a light microscope with phase contrast optic. The gold layer did not impair the light microscopic viewing. The area surrounding the chromosomes intended for SEM study was cut down to the respective size of the SEM specimen holder using a diamond tipped glass cutter and was affixed to the specimen holder using a double-sided tape. The edges of the cover slip were then sealed and connected to the specimen holder with carbon paste. The chromosomes were examined in a Jeol 35 scanning electron microscope at 30 kV using magnifications of 100, 5,000 or 10,000x.

4.7. Laser scanning microscopy (LSM) and conventional differential interference contrast (DIC) in incident light microscopy of SSP chromosomes

The Laser Scan Microscope (LSM) was a prototype of a scanning light microscope which used the principle of beam scanning in contrast to object scanning which is the principle used in the confocal scanning microscope of Brakenhoff (1979) and Brakenhoff et al. (1979). A detailed description of the LSM was given elsewhere by Wilke et al. (1983). Therefore, only the main features are described here.

An expanded laser beam (Ar⁺-laser, wave length = 488 nm) is raster scanned by two servo-controlled galvanometer scanners. The scan mirrors are imaged into the pupil of the

microscope objective in order to prevent beam truncation during the scanning process. The full aperture of the objective is used in this way independent of the scan angle. The size of the spot projected onto the specimen by the objective is diffraction limited. The spot diameter can be smaller than 0.5 μm for high aperture objectives. The full scan angle is approximately 3.5° . In this way, zooming of up to a factor of 8 is achieved. The frame time is variable between 2 sec and 1 min. The image may consist of 512×512 or 1024×1024 picture elements.

Photomultipliers serve as detectors for light transmitted through or reflected by the specimen. Contrast and brightness of the image can be controlled in a wide range by changing the photomultiplier gain and by subtracting DC levels from the amplified signal.

SSP chromosome preparations of Chironomus were prepared and picked up subbed slides as described in section 4.1.

The conventional DIC pictures were taken in incident light with a Zeiss photo microscope. For the illumination, monochromatic light from a mercury lamp (Osram HBO 100) was obtained with an interference line filter (wave length = 546, 11 nm FWHM).

The objectives Epiplan 40/0.85 Pol and Epiplan 80/0.95 Pol with the highest aperture available for dry objectives were used for the incident light DIC pictures. For the LSM phase contrast pictures, the objective Planapo 40/0.95 Ph 3 was chosen in order to maintain the same laser spot size as for the DIC pictures. Photographs were taken on Polaroid film from off a high resolution monitor at about 400x and 800x, respectively. The pictures shown are enlargements of the Polaroid originals.

5. RESULTS

[The sources on which individual sections are based on, are denoted at the beginning of each chapter or section by roman numerals which refer to the respective publications listed on page V following the Table of Contents. The term "unpublished" indicates those sections or chapters comprised of completely new material, none of which has previously appeared elsewhere in any form whatsoever.]

5.1. Introductory observations

(Sources: 1a based on IV; 1b,c,d,e based on Whitmore, unpublished; 2 based on V; 3 based on Whitmore, unpublished)

Whole mount electron microscopy of dipteran polytene chromosomes utilizing the surface-spread polytene (SSP) chromosome preparation technique (see Methods section 4.1.) offers three distinct advantages over routine light microscopy of squash preparations.

(1) High resolution depictions of chromosomal characteristics or peculiarities

Such as:

- (a) The banding pattern - Figure 3 shows as an example, a comparison between an electron micrograph of a single SSP chromosome preparation of salivary gland chromosome 3L (divisions 71 - 80) of Drosophila melanogaster and the LM reference map of Lefevre (1976) which was a composite of the best sections from several chromosomes.
- (b) Ectopic pairing - Ectopic pairing is in most cases the conjugation between different chromosomes or sections of an individual chromosome with threads of DNP material. It is said to be a characteristic of intercalary heterochromatin along with late replication and local underreplication (Gvozdev, 1981). One can differentiate between ectopic pairing in which separate bands of nonadjacent positions are connected and ectopic pairing between bands of fairly adjacent positions. Whereas the first type can be recognized light microscopically, the latter can be only detected with confidence using the higher resolution of the electron microscope (Zhimulev et al., 1982). Figure 4A is an

electron micrograph of Malpighian tubule chromosome I in Chironomus thummi piger showing ectopic pairing of the both types. Figure 4B is a cut-out of "4A" depicting ectopic pairing of the first type between separate bands of nonadjacent positions in divisions B1 and B3 at a higher magnification. Figure 4C is a cut-out of "4A" depicting the ectopic pairing of the second type between bands of fairly adjacent positions in division C2 at a higher magnification.

(c) **The chromocenter region** - In contrast to most species of Chironomus, the majority of Drosophila species show adhesion of the heterochromatic centromeres of the chromosomes forming a mutal so-called "chromocenter" (Beermann, 1962). It is here that the completely underreplicated Y-chromosome and α -heterochromatin are to be found. Very little is actually known concerning the further nature of the chromocenter (reviewed in Richards, 1985). Figure 5 depicts the chromocenter of Drosophila hydei in an electron micrograph.

(d) **Nonhomologous regions** - In most Diptera, the homologous paternal and maternal chromosomes remain paired during the formation of the polytene chromosomes. At areas of heterozygous deletions, insertions, inversions, or other structural differences, the chromosomes can remain unpaired depending on the structural length of the chromosomal mutation. Such chromosomes are usually of poorer cytological quality than normal chromosome preparations. This phenomenon can be observed, however, for entire chromosomes at high resolution using SSP chromosomes. In Figure 6, an electron micrograph is shown of chromosome II from a Chironomus thummi thummi x Chironomus thummi piger hybrid. Here, the chromosomal pairing is restricted to some sections towards the end sections of the chromosomes. The central sections of the chromosomes remain unpaired. A result, most likely, of the evolutionary divergence of

the two species which has led to a number of structural differences in these sections (Keyl and Strenzke, 1956).

(e) **Puffs**- Puffs are decondensations of polytene chromosome bands or band/interband groups which indicate areas of high transcriptional activity (Pelling, 1964). Figure 7 depicts an electron micrograph of a stage specific puff seen in Chironomus th. thummi chromosome I in late fourth instar larvae.

(2) SSP chromosome bands are extended well enough to be measured individually

Figure 8 shows an example of the measurement of band thicknesses and interband lengths in a well-extended SSP chromosome. On the basis of the mode of measurement shown, the banding pattern of entire chromosomes can be digitized and computer plotted as described in Methods section 4.2.

As an example, the band-interband pattern of chromosome I of Chironomus th. thummi was digitized in three comparable, well-extended SSP chromosome preparations (one of which is depicted in Figure 9C). The average values for homologous data were then plotted. Figure 9B shows a cutout of this computerized EM map for divisions F1-4. Figure 9A shows the band-interband pattern of the same region from the hand-drawn chromosome map of Hägele (1970). [Several chromosome bands (for example, F2k, F2l, and the telomere) show flared borders on one or both sides (including stage specific decondensation of polytene structures). These need comparable analyses of homologous structures from a number of SSP chromosome preparations.]

Although there are differences in individual thicknesses and interband lengths, differences in the number of bands is small, if one considers that Hägele's map is

based on LM analyses of many squash preparations. The original Chironomus strain (A62), on which the Hägele's map is based, no longer exists. Therefore, pattern differences shown in Figure 9 could be at least partially based on two slightly different genetical backgrounds. This also indicates another advantage of the SSP method in being able to obtain in a reasonable time high resolution depictions of the chromosome banding patterns of new stocks or strains.

(3) The possibility of observing the structural organization of the interbands and bands

In Figure 10A, a stretch of several bands and interbands are shown at a high magnification from Malpighian tubule chromosome II of Chironomus th. piger.

The chromatin material appears in the heavy bands at the top as an electron dense amorphous complex of deoxyribonucleoprotein (DNP) fibers. These bands have thicknesses of ca. 0.5 - 1.4 μm and are separated by interbands of ca. 0.2 - 0.6 μm . The thick lower band is followed by a long interband of ca. 2.5 μm which precedes two slightly decondensed bands. The elementary DNP fibers can be seen in the interband region as running approximately parallel to the axis of the chromosome with widths varying from ca. 10 - 30 nm. The majority of the fibers have a 20 - 25 nm thickness with an irregular granular type structure.

A general impression of the state of DNA decondensation in the interbands can be obtained from a higher magnification. In Figure 10B, a cutout is shown by ca. 240,000x of an interband section from the subdivision denoted as E2 in Figure 10A. Indicated by arrows are: (a) thin, presumably, DNP fibers of ca. 3.5 - 5 nm thickness with beaded type structures. These types of fibers fill the lighter gray portions of the interband region and can also be seen on

the periphery of the chromosome itself. Fibers of this morphology have been described earlier by Alanen (1981) in an integrated study of Drosophila and Chironomus polytene chromosomes. (b) A row of tightly synapsed, spherically shaped particles of ca. 4.5 - 6 nm. (c) Apparently, a slightly flattened and separated version of "b" with more of an elliptical shape of ca. 6 x 8 nm. Judging from the general size and shape of "b" and "c", these particles are most likely examples of nucleosome structures. (d) An interband fiber of ca. 20 - 25 nm consisting of irregular rows of ca. 4 - 6 nm particles attached to ca. 2 - 2.5 nm fibers. (e) Apparently, a further condensation of an interband fiber into a globular structure of ca. 30 nm consisting of ca. 12 - 20 particles of ca. 6 nm size. This structure is similar to the one described as a "superbead" in a study of salivary gland chromosomes of Chironomus tentans by Sass (1980).

Some bands, especially in Drosophila species, may appear as dotted structures rather than as the more or less straight lines transversing the width of the chromosomes as depicted above in the chromosome in Figure 10. Figure 11A shows an electron micrograph of one such section from the X chromosome of the Drosophila hydei salivary glands. The area indicated in this micrograph is depicted at a higher magnification in Figure 11B. Here, the 20 - 30 nm interband fibers (interchromomeres) are grouped into cable-like bundles which fork shortly before entering the banded material (chromomeres). The chromatin material of the band on the border between 17B and 17C is visibly embeded in an amorphic protein complex. The complex honey-comb like substructure of the chromomeres is clearly visible in the band directly underneath in division 17B.

In viewing the cytologic material, one must take into consideration the possible influence of the methodology itself on the chromosomal substructures, i.e. the production of artifacts etc. The same is true with any of the

chromosome preparation methods presently available. Although, it is assumed that the chromosomal spreading effect seen in the SSP chromosome preparation technique is a composite of chromosomal protein depletion by the propionic- and citric acid pretreatment and the physical forces exerted on the chromosomes by the spreading process, there is no evidence available concerning the actual extent of the protein depletion. In fact, Alanen (1981 and references therein), using a quite similar methodology, stated that "there is no reason to expect that the methods used would remove significant amounts of protein from the chromatin".



Figure 3. Salivary gland chromosome divisions 71 - 80 (3L) of *Drosophila melanogaster*. A comparison between an electron micrograph of a single SSP chromosome preparation and the LM reference map of Lefevre (1976). Under the assumption that the squash preparation represents a chromosome diameter of 5 - 8 μm , then both chromosomes show the same magnification. It should be noted that Lefevre's reference map is a composition of several well-extended chromosome preparations. By this, the longitudinal (axial) degree of spreading of the SSP chromosome appears reduced from what is normally observed in comparison with routine squash preparations. Bar represents 10 μm . (Source: IV)



Figure 4A. An electron micrograph of Malpighian tubule chromosome I in *Chironomus thummi piger* depicting "ectopic pairing" between (a) separate bands of nonadjacent positions and (b) bands of fairly adjacent positions. Arrows indicate intercalary chromatin threads. 2,800x. (Whitcomb, unpublished)



Figure 4B. Cut-out from Figure 4A depicting the "ectopic pairing" between separate bands of nonadjacent positions at a higher magnification. 5,600x. Arrow indicates intercalary chromatin threads. (Source: Whitmore, unpublished)

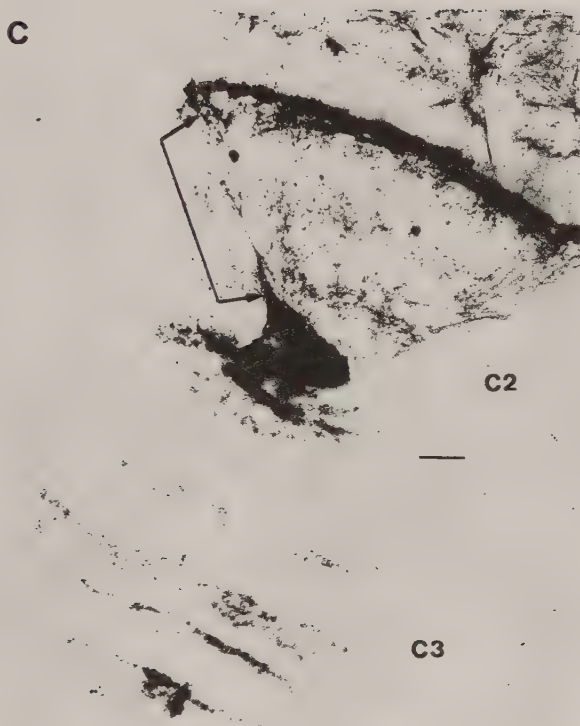


Figure 4C. Cut-out from Figure 4A depicting the "ectopic pairing" between bands of fairly adjacent positions. Arrows indicate intercalary chromatin threads. 5,600x. (Source: Whitmore, unpublished)

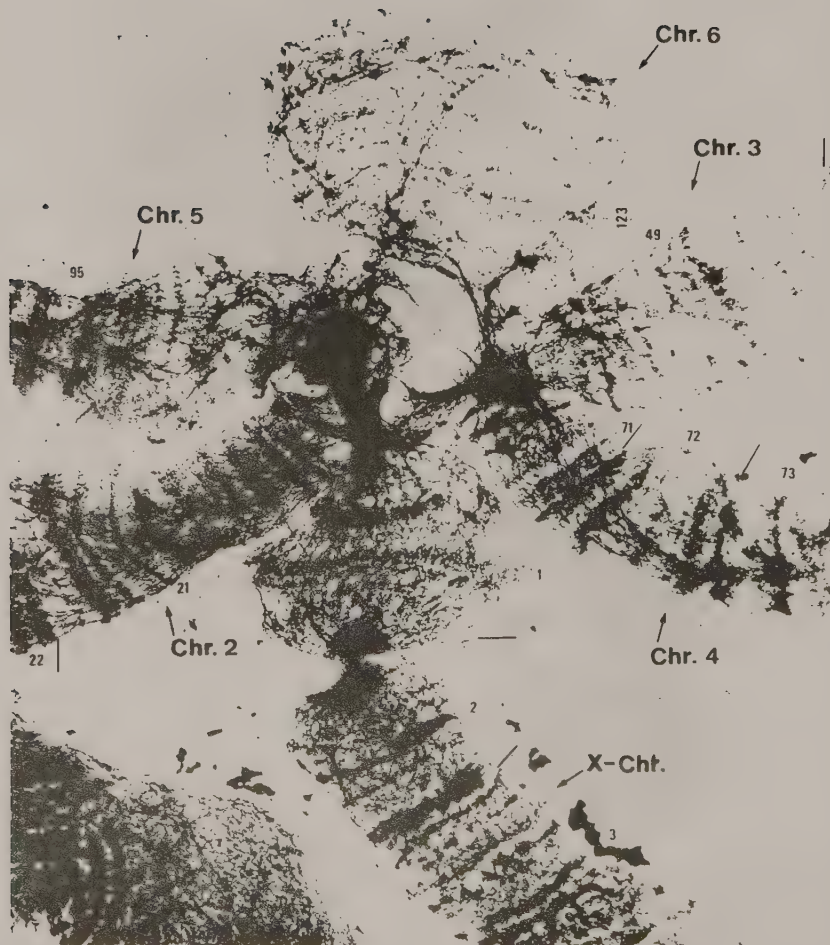


Figure 5. The "chromocenter" region of *Drosophila hydei* depicted in an electron micrograph. Reference numbers according to Berendes (1963). 4,480x. (Source: Whitmore, unpublished)



Figure 6. An electron micrograph of chromosome II from a Chironomus th. thummi x Ch. th. piger hybrid. Note that the central regions of the homologous chromosomes are not somatically paired. Arrows indicate the centromeric region by piger (pi) and thummi (th). 570x. Bar indicates 10 μ m. (Source: Whitmore, unpublished)

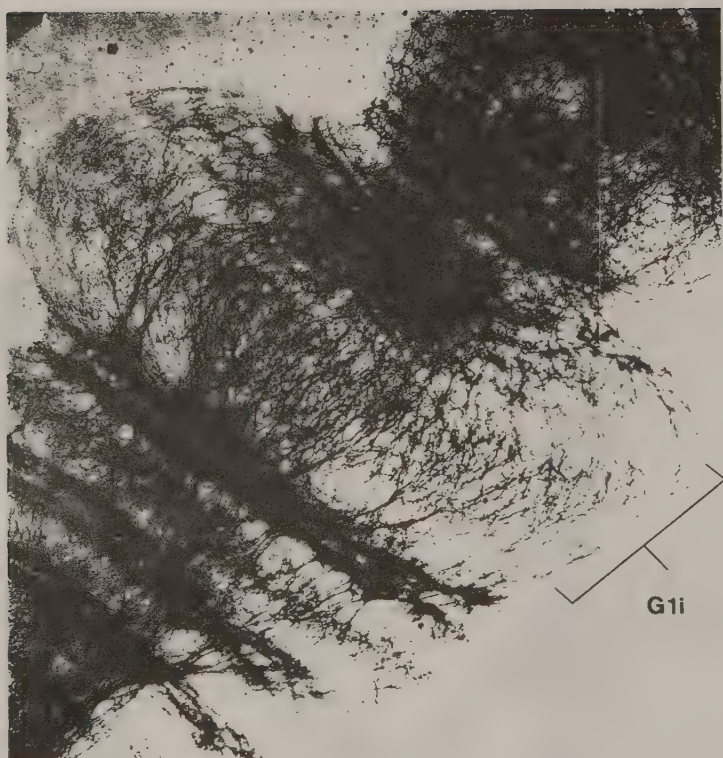


Figure 7. An electron micrograph of a stage specific puff seen in Chironomus thummi thummi chromosome I, division G1i, in late fourth instar larvae. Reference number according to Hägele (1970). 3,200x. (Source: Whitmore, unpublished)

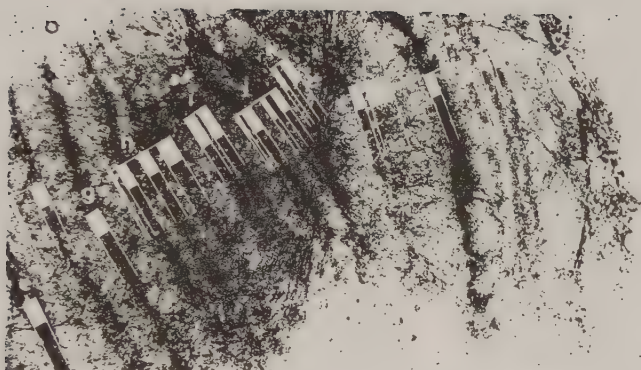


Figure 8. An illustration of the measurement of the band thicknesses and the interband lengths in an electron micrograph of a SSP chromosome. Telomeric region (F2) of the left arm of salivary gland chromosome II in Chironomus th. thummi. Reference numbers according to Hägele (1970). 3,200x. (Source: V)

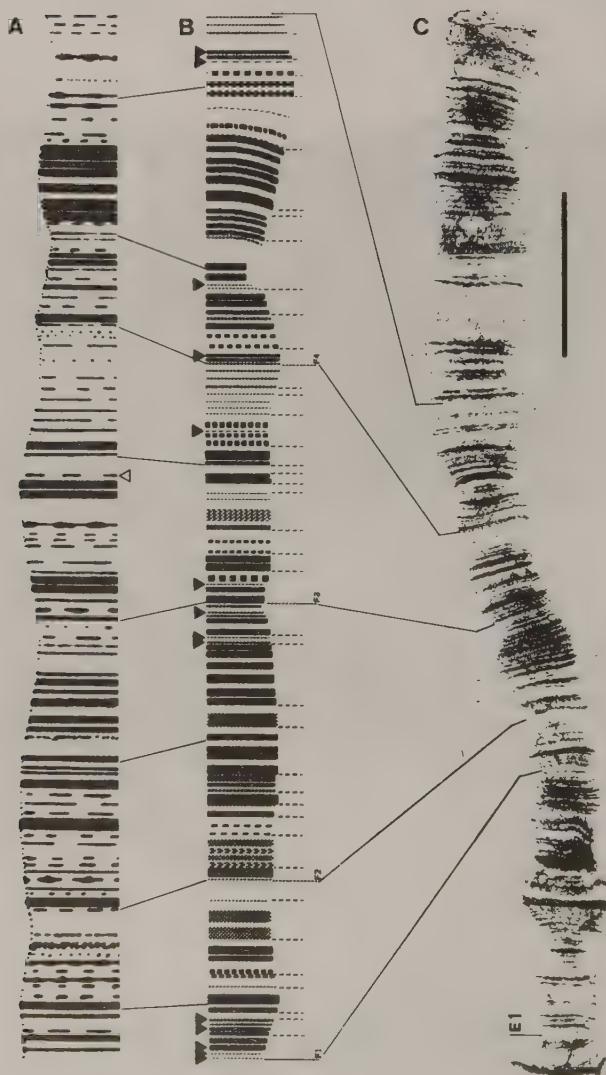


Figure 9. A comparison of the band-interband pattern of the left arm of salivary gland chromosome I in *Chironomus thummi thummi* between (A) a hand-drawn chromosome map of the F1 - 4 region based on LM analyses of a large number of squash preparations (Hägele, 1970), (B) a computerized chromosome map based on measurements and digitizing from electron micrographs of three SSP chromosomes with comparable degrees of spreading, and (C) an electron micrograph of one SSP chromosome preparation. 600x. The white arrow in "A" indicates the only band not found so far in SSP chromosome preparations. Black arrows in "B" indicate additional structures found in SSP chromosomes. (Source: U)



Figure 10A. An electron micrograph of the structural organization of the bands and interbands at a high magnification from Malpighian tubule chromosome II of Chironomus th. piger. 15,500x. (Source: Whitmore, unpublished)



Figure 10B. Ultrastructural electron microscopic (EM) depiction of the state of chromatin condensation in the interband section denoted as subdivision E2 in Figure 10A. 242,850x. Arrows indicate (a) thin, presumably, DNP fibers of ca. 3.5 - 5 nm thickness with beaded type structures, (b) a row of tightly synapsed, spherically shaped particles of ca. 4.5 - 6 nm, (c) apparently, a slightly flattened and separated version of "b" with more of an elliptical shape of ca. 6 x 8 nm (presumably nucleosome structures), (d) an interband fiber of ca. 20 - 25 nm consisting of irregular rows of ca. 4.5 - 6 nm particles attached to ca. 2 - 2.5 nm fibers, (e) apparently, a further condensation of an interband fiber into a structure of ca. 30 nm. (Source: Whitmore, unpublished)



Figure 11A. The structural organization of the bands and interbands as seen in an electron micrograph of division 17 of the X chromosome in *Drosophila hydei*. Reference numbers according to Berendes (1963). 15,600x. (Source: Whitmore, unpublished)

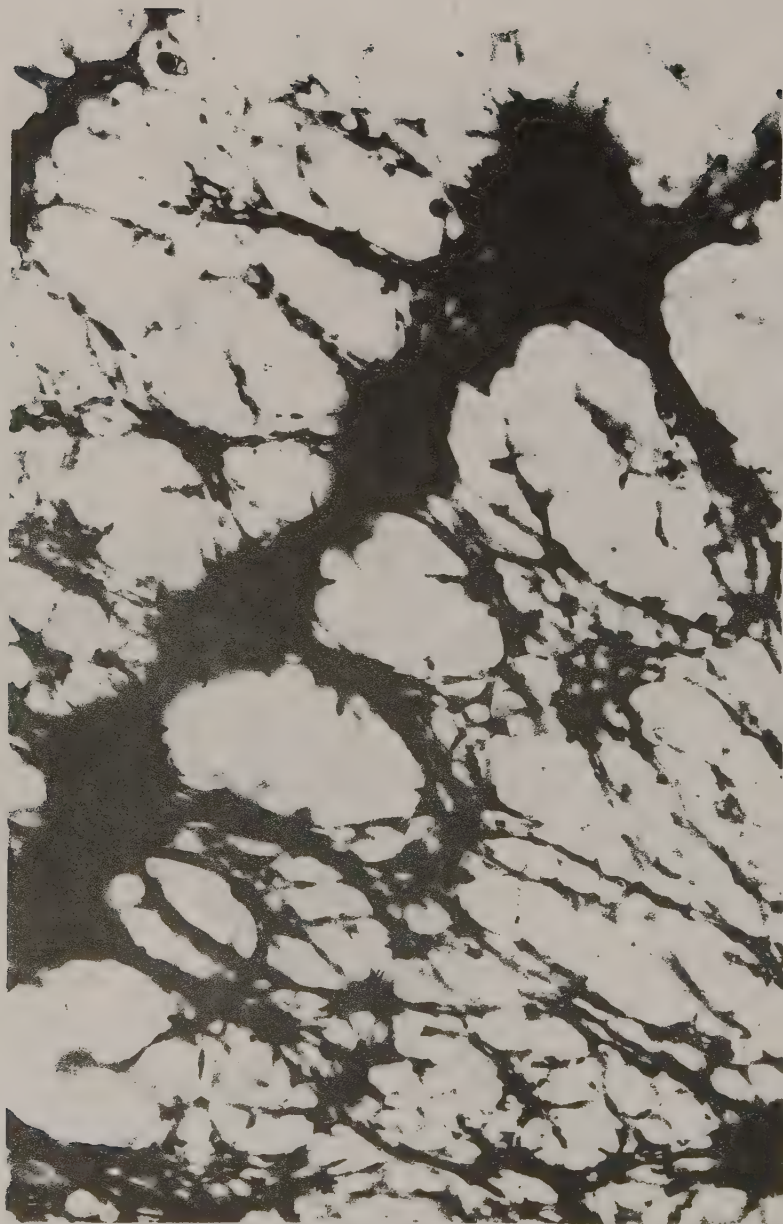


Figure 11B. A cut-out of the area indicated by white brackets in Fig. 11A at a higher magnification. 52,000x. (Source: Whitmore, unpublished)

5.2. Electron microscopic photo maps of surface-spread polytene (SSP) chromosomes in Drosophila hydei

(Sources: based on VI, VII, and XV)

In this section, an electron microscopic (EM) photo map is shown covering the entire genome of Drosophila hydei. It should be emphasized that here the intention was to give an overview of the chromosomes for practical mapping purposes rather than attempting to depict the total number of bands in individual subdivisions of the pattern.

Chromosome selection and labeling: It was attempted to select: straight chromosomes (or at least longer sections without superpositions), chromosomes without "ectopic pairing", adhesion of the telomeres, or breaks in potentially underreplicated sections, chromosomes with high lateral and longitudinal degrees of spreading, chromosomes with comparable degree of polytenization, and chromosomes at comparable stages of larval development. It was difficult to obtain chromosomes that fulfilled all of these requirements as compared with salivary gland chromosomes from Chironomus species (see Kalisch et al., 1984). Therefore, the chromosomes depicted in Figures 12 - 15 were composed from several SSP chromosome preparations.

Furthermore, the chromosomes in Figures 12, 14, and 16 were also cut several times before being assembled. Segmentation and labeling of the divisions and subdivisions of the entire genome was based on those of Berendes' map (1963). Micrographs of chromosome 4 (subdivisions 86A - 94C) and of chromosome 6 were also used in computer studies of EM chromosome mapping (Reiling et al., 1984a,b; Whitmore et al., 1984). A summary is provided in Table 1 of other cytological studies of Drosophila hydei.

X chromosome in Drosophila hydei

The EM photo map of the X chromosome (Figure 12) was composed of five different SSP chromosome preparations: 1A-5B, 5B-9A, 9A-14A, 14B-18B, and 18B-20D. The total number of bands depicted is about 500. This is about a 43% increase (500:350) compared with the LM chromosome map of Berendes (1963). The total number of bands in individual subdivisions of the X chromosome are given in section 5.2. of Results together with an EM chromosome map.

A comparison of the EM photo map with the LM photo map of Ananiev and Barsky (1982) shows differences in the sectioning of the left borders of the following X chromosome divisions: 3, 5, 9, 11, 13, and 16.

Differences are also found in the sectioning of the left borders of subdivisions: 8C-10A, 15D, 16C-D, 17D, and 18D in the chromosome and photo maps of van Breugel et al. (1986). For the 16A-D region there exists, however, a revised sectioning in the LM photo map of van Breugel and van Zijl Langhout (1983).

The X chromosome from the SSP preparation has a total length of ca. 360 μm (excluding the overlapping sections in Figure 12). The corresponding data for the X chromosome in squash preparations is $157 \pm 25 \mu\text{m}$. The average diameter of SSP chromosomes in Figures 12 - 16 is $7 - 25 \mu\text{m}$ compared with $2.2 - 8 \mu\text{m}$ in squash preparations (Berendes, 1963).

Chromosome 2 in Drosophila hydei

The EM photo map of chromosome 2 (Figure 13) was composed of two chromosomes containing the sections: 21A-29A and 28C-48C. It has a total length of about 400 μm in comparison with $184 \pm 20 \mu\text{m}$ in squash preparations

(Berendes, 1963). For 28C-48C a chromosome is depicted with a lower degree of polyteny as well as lower degrees of lateral and longitudinal spreading.

There are differences in the sectioning of the left borders of the following divisions in comparison with the LM map of Ananiev and Barsky (1982): 24, 28, 36, 37, 41, 43, 46, and 47. A comparison between chromosome 2 (Figure 13) and an electron micrograph of a thin-sectioned squash preparation of region 35A-48C (Figure 3 in Grond and Derksen, 1983) indicates that on the average more cytological details can be depicted in an individual SSP chromosome.

However, it should also be noted that the total number of bands registered in that chromosome map based on EM analyses of several chromosome preparations (Figure 4 in Grond and Derksen, 1983) shows more structural details than in this study. In the same paper, differences are to be found in the subdivision sectioning in comparison with Berendes' map (1963): 35C, 36A, 38C-D, 41C-D, 46D, and 47B.

There are also differences between Berendes' map and the LM chromosome map as well as the confocal scanning LM photo map of Grond et al. (1982) in : 46C-D, 47A, and 48C. This could be caused, at least partly, by differences in the reproduction of the total number and prominence of bands in subdivisions 48A-C of Berendes' map in that paper. Finally, in comparison with Berendes' map (1963), the following minor differences in the sectioning are to be found in van Breugel et al. (1968): 22B, 25A-26A, 26B-C, 27A-D, 28A (Figures 3 and 4 in that paper); 32B-C, 33D (Figures 4 and 5); 35A-36B (Figures 4 and 6); 38B-C, 39B-D (Figure 5); and 47D (Figure 6).

Chromosome 3 in Drosophila hydei

The photo map of chromosome 3 is composed of four different SSP chromosome preparations: 49A-56C, 55D-64B, 63A-70D (excluding 66C) and 66C (Figure 14). The following differences exist for the sectioning of the left division borders in comparison with the LM map of Ananiev and Barsky (1982): 56-58 and 62.

The distal part of chromosome 3 (56D-70D) was the most difficult region of the genome to prepare because of three threadlike constrictions (56C, 66C, and 66CD; see also Table 2); 56C was always broken in the SSP chromosome preparations. Due to this, the distal part of the chromosome separated during the spreading process from the rest of the genome and usually showed (if found at all) superpositions and a lower degree of spreading than the rest of the genome. In the SSP chromosome preparations depicted in Figure 14, "55D-64B" are two broken parts of the same chromosome.

Subdivision 66C does not usually show its banding pattern distinctly because of the two, more or less, threadlike constrictions (66C and 66CD), which favor a pattern disorder of the flanking subdivisions. It has been tried to insert the 66C from a SSP chromosome preparation in which a pattern could be found comparable with that in Berendes' map. The threadlike superposition crossing subdivisions 59AB does not belong to chromosome 3. The total length of chromosome 3 is 530 μm in comparison with $150 \pm 40 \mu\text{m}$ in squash preparations (Berendes, 1963).

Chromosome 4 in Drosophila hydei

The SSP chromosome 4 has been reconstructed from two different preparations: 71A-85C and 85C-94C (Figure 15). A cytological analysis of seven SSP chromosomes

showed an approximately 40% increase (206:147) of chromosomes bands for region 86A-94C in comparison with Berendes' map (Reiling et al., 1984b). Due to decondensed chromosome structures, the subdivision sectioning for 76AB and 78AB in Figure 15 was arbitrary. The total length of the SSP chromosome is 330 μ m compared with 149 ± 25 μ m in squash preparations (Berendes, 1963).

A comparison between Berendes' map and the LM photo map of Ananiev and Barsky (1982) showed differences for the left division borders in: 74, 75, 77, 80, 82-84, 90, and 92.

Chromosome 5 in Drosophila hydei

The EM photo map of chromosome 5 represents a single SSP chromosome preparation (Figure 16). Its total length is 330 μ m in comparison with 160 ± 25 μ m in squash preparations (Berendes, 1963). Pattern sectioning of chromosome 5 is difficult due to several puffs which occur at the end of the third larval instar.

Berendes (1965, 1966) had already tried to revise this chromosome several times by mapping different stages of larval development. Between the EM photo map and the LM photo map of Ananiev and Barsky there are the following differences to be found in the left division borders of: 96-102; 105, 107, 113, 114, 118, and 119.

Chromosome 5 shows several characteristic constrictions in SSP chromosome preparations (compare Table 2). Subdivisions 102B-D could not be spread properly. In one case some spreading was obtained with a strong "ectopic pairing" between the prominent bands of this division. Chromosome sections 98D (not in Figure 16), 109B, and 121C could be threadlike, whereby 109B was

often broken and showed "ectopic pairing" between 109B and 110A as depicted in Figure 16.

According to the puffing activity, especially in the distal part of the chromosome, the appearance of the fine polytene structures can vary considerably in SSP chromosomes (for comparison see also Berendes 1965, 1966). This is also true for 121AB, which can have different numbers of very thin bands in different SSP chromosome preparations. For these subdivisions, an EM chromosome map must be based on many detailed EM analyses of SSP chromosomes to show accurately the total number of polytene structures involved.

Chromosome 6 in Drosophila hydei

The chromosome 6 shown in Figure 17 is the same one used for a computerized EM chromosome map having 30 chromosome bands compared with 15 bands in Berendes' map (Whitmore et al., 1984). The length is 10 μ m in comparison with $5 \pm 0.5 \mu$ m in squash preparations (Berendes, 1963).

Chromosome constrictions: Chromosome outlines of all the SSP chromosomes prepared so far from Drosophila hydei were analyzed with respect to prominent constrictions. The data were compared with those of squash preparations from the literature listed in Table 1. Although there is a high degree of correspondence in chromosome morphology for the two techniques, there are also some characteristic differences. Table 2 lists the most prominent constrictions in SSP chromosomes and squash preparations.

In SSP chromosomes one can differentiate two types of constrictions. Type-1 constrictions are found exclusively at distinct bands or band groups. This type is assumed to be caused by differential distribution of chromosomal proteins. The prominence of type-1 constrictions decreases,

however, as the degree of lateral chromosome spreading increases. Type-1 constrictions were also less prominent in chromosomes that had been subjected to a long acid pretreatment. Most type-2 constrictions are to be found in interbands or sections with faint chromosome bands, but type-2 constrictions can also be found adjacent to prominent bands (for example, 1D-2A and 85BC). Characteristically the type-2 constrictions are threadlike or can even break in SSP chromosomes. It is assumed that this type represents under-replicated chromosome sections.

One has to distinguish between threadlike chromosome sections in squash preparations where the mechanical stretching of the chromosomes results in a diminution of the chromosome diameter and threadlike constrictions in SSP chromosomes. While almost any chromosome section can be stretched threadlike in squash preparations, SSP chromosomes show this behavior only at specific points. Nevertheless, whether a type-2 constriction is threadlike in an individual SSP chromosome preparation probably also depends on the degree of longitudinal spreading.

One explanation for the appearance of type-1 constrictions in SSP chromosomes could be quantitative or qualitative differences in chromosomal proteins of the chromosome bands involved. These differences could be the result in a time-dependent reduced reaction with the pre-treatment solution compared with the rest of the genome. The type-2 constrictions seem to represent, however, underreplicated sections. This appears to be the case for 1D-2A from recent investigations concerning extranucleolar rDNA introns in D. hydei (Kunz, personal communication).

The constrictions of squash preparations have been listed separately in Table 2 because of the differences existing between photo and chromosome maps. It has not been investigated, however, whether the differences between photo and chromosome maps in squash preparations are caused by

methodological reasons that may be comparable to the causes of variable prominence of the type-1 constrictions in SSP chromosomes.

Table 1. Literature containing cytological data on salivary gland chromosomes of *Drosophila hydei*, used for comparison and for the pattern sectioning in Figures 12-17

Region	Reference
1. LM chromosome maps	
1A-123F	Berendes 1963
7A-10A; 15D-17D	van Breugel et al. 1968
16A-17C	van Breugel and van Zijl Langhout 1983
18A-19C; 21C-23D	van Breugel et al. 1968
24A-26D; 25A-28A	van Breugel et al. 1968
30D-33D; 32D-35D	van Breugel et al. 1968
35A-36C; 38B-40B	van Breugel et al. 1968
47A-48C	van Breugel et al. 1968
46A-48C	Grond et al. 1982
102A-103C	van Breugel et al. 1968
112B-119D; 115-119	Berendes 1965
117A-122D	Berendes 1966
2. LM photo maps	
1A-20D	Whitmore and Kalisch 1984
1A-123F	van Breugel, unpublished
1A-123F	Ananiev and Barsky 1982
15D-17D	van Breugel et al. 1968
15D-17D	van Breugel and van Zijl Langhout 1983
17D-20D	Kalisch 1982a
46A-48C	Grond et al. 1982
46D-48B	Whitmore and Kalisch 1984
93-94	Kalisch and Hägele 1982
3. EM chromosome maps	
35A-48C	Grond and Derksen 1983
48C	Berendes et al. 1974
83A-85C	Reiling et al. 1984a
86A-94C	Reiling et al. 1984b
123A-F	Whitmore et al. 1984
4. EM photo maps	
Telomers	Berendes and Meyer 1968
35A-48C	Grond and Derksen 1983
48A-C	Berendes 1972
86A-94C	Reiling et al. 1984b
123A-F	Whitmore et al. 1984

Figure 12. Electron microscopic map of salivary gland X chromosome of Drosophila hydei. 1,000x.
(Source: VI)



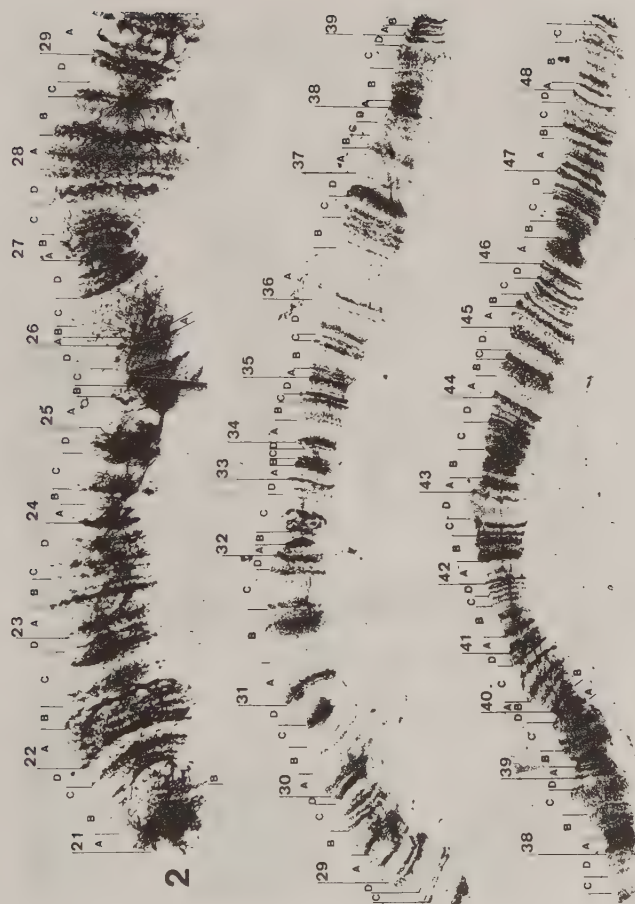


Figure 13. Electron microscopic map of salivary gland chromosome 2 of Drosophila hydei. 1,000x.
(Source: VI)



Figure 14. Electron microscopic map of salivary gland chromosome 3 of Drosophila hydei. 1,000x.
(Source: VI)

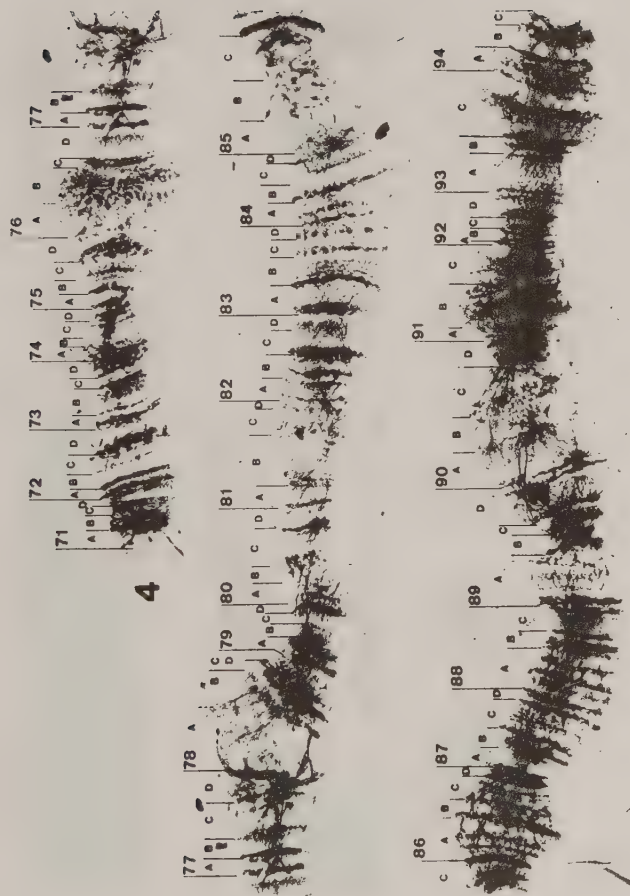


Figure 15. Electron microscopic map of salivary gland chromosome 4 of *Drosophila hydei*. 1,000x.
(Source: VI)

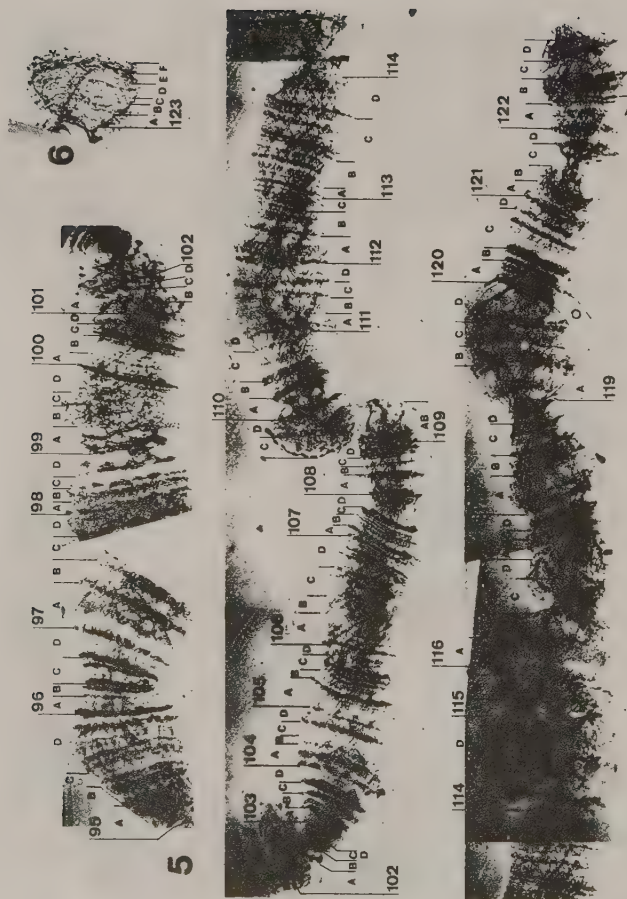


Figure 16. Electron microscopic map of salivary gland chromosome 5 of Drosophila hydei. 1,000x.
 Figure 17. Electron microscopic map of salivary gland chromosome 6 of Drosophila hydei. 1,000x.
 (Source: VI)

Table 2. Prominent chromosome constrictions of surface-spread polytene (SSP) chromosomes in comparison with homologous sections in squash preparations as listed in Table 1 (Type-1, constrictions at prominent chromosome bands or groups of bands; type-2, constrictions (sometimes threadlike) at interbands or sections with faint chromosome bands)

	SSP chromosomes ^a		Squash preparations ^b	
	Type-1	Type-2	Photo maps	Chromosome maps
<i>X</i> chromosome				
1D-2A	+	+	+	+
5A-C	+	-	-	-
8D-9A	+	-	-	-
11AB	+	-	+	+
17A	+	-	+	(+)
Chromosome 2				
24A	+	-	+	+
25A	-	+	-	+
27A	+	+*	+	-
29CD	+	-	+	+
30CD	+	-	+	+
32B	+	+*	(+)	(+)
38AB	+	-	(+)	-
39B	+	-	+	(+)
43B	+	-	-	+
46BC	+	-	-	+
48B	+	-	+	+
Chromosome 3				
51AB	+	-	+	+
56C	-	+	+	-
64B	+	-	-	-
66C	-	+	+	-
66CD	+	+*	+	-
67C	+	-	+	+
68C	+	-	+	+
Chromosome 4				
73C	+	-	+	+
85BC	+	+	+	+
89B	+	-	-	-
90CD	+	+	-	+
Chromosome 5				
98D	+	+	-	+
102BD	+	-	-	-
109B	-	+	-	+
110A	+	-	+	(+)
116C	+	-	+	+
118D	+	-	+	-
121C	+	+	+	+

^a Data based on all SSP chromosomes of *D. hydei* analysed so far by Kalisch, Reiling, Whitmore and coauthors (Table 1). Asterisks indicate that the type-2 constriction has been found so far to be threadlike only once

^b Data from literature listed in Table 1. Parentheses indicate that the constriction is not prominent

5.3. Computerized electron microscopic chromosome mapping of chromosomes X, 4, and 6 in Drosophila hydei

An electron microscopic fine analysis was made of the band-interband patterns of salivary gland chromosomes X, 4, and 6 in Drosophila hydei by electron microscopy (EM) using surface-spread polytene chromosome preparations. Band thicknesses, band diameters, and interband lengths of individual polytene structures were measured on electron micrographs of six to ten SSP chromosome preparations. Data of homologous structures were computerized for calculating average values and for plotting the EM chromosome maps. A Sharp PC-1500 pocket computer and a Sharp CE-150 plotter were used for digitizing and plotting of chromosome 6. An IBM PC XT and a HP7475A plotter were used for chromosomes 4 and X (see Methods section 4.2. for more details).

The presentation given here, follows chronologically that of the program development (chromosomes 6, 4, and X, in that order).

5.3.1. Chromosome 6 in Drosophila hydei

(Sources: based on XIV and Whitmore, unpublished data)

Pattern analysis: Among the various Drosophila species probably the most difficult polytene chromosomes to investigate are the so-called "dot" or small rod shaped chromosomes. In contrast to the relatively well defined banding pattern of longer chromosomes these extremely short chromosomes are characterized by their faint and unclear bands. To complicate the problem further, they are usually attached to or embedded in the chromocenter along with the proximal ends of the other chromosomes.

Berendes (1963) describes chromosome 6 of D. hydei as being partly heterochromatic and consisting of 17 concen-

tric bands. In the same study he chose to depict, however, only 15 bands in his chromosome map. The chromosome's shape is variable, but usually takes on a top- or rod-like configuration. Based on the data obtained from the analysis of 6 EM micrographs, 30 bands were included in the computerized map (Figure 18). Four additional bands were also seen once or twice, but were not included in this map. If they had been, this would have brought the total number of bands up to a possible 34. Either way, this is an approximate 100% increase in the total number of chromosome bands compared to the original light microscopic chromosome map of Berendes based on squash preparations.

Quantitative analysis: Originally, the measurement of individual structures in SSP chromosomes was undertaken to enable a better correlation between chromosome cytology and chromosome maps (Kalisch et al., 1984). However, those data can also be used not only to obtain information about the spreading process in homologous structures, but also for a detailed analysis of interband lengths and band thicknesses in SSP chromosomes. Examples of the methods used to quantify individual band thicknesses as well as interband lengths in electron micrographs were published in Kalisch et al. (1984).

The average axial interband length calculated for chromosome 6 was 0.18 μm . The standard deviation was $\pm 0.10 \mu\text{m}$, i.e. ca. 2/3 of the interbands are in the range of 0.08 μm to 0.28 μm . Due to the transitional nature of SSP chromosome preparations, an exact DNA packing ratio in the interbands cannot be calculated by cytological measurements of the diameters of the interband fibers as has been done in comparable investigations (Sorsa, 1982a,b). Total extension of the DNA (0.34 $\mu\text{m}/\text{Kb}$) can be assumed, nevertheless, as a minimum DNA length within a given interband. Analyses of higher degrees of chromosome spreading (Kalisch et al., 1985b) indicate, however, that this total extension of DNA is generally not realized in all the interbands of SSP

chromosomes. Several authors, using various chromosome preparation techniques, have reported that nucleosome structures exist both in bands and interbands (Sass, 1980; Alanen, 1981; Ananiev and Barsky, 1985). Kress et al. (1985) calculated a DNA packing ratio of $0.10 \mu\text{m}/\text{Kb}$ for the interbands of SSP chromosomes from molecular data in Drosophila.

If one accepts $0.34 \mu\text{m}/\text{Kb}$ as a minimum and $0.10 \mu\text{m}/\text{Kb}$ as a maximum DNA packing ratio in the interbands of SSP chromosomes, then the average interband of chromosome 6 ($0.18 \mu\text{m}$) represents a DNA length of between 0.5 kb and 1.8 kb. Due to the standard deviation of $\pm 0.10 \mu\text{m}$ mentioned before, ca. 1/6 of the interbands analyzed are on the average $\leq 0.08 \mu\text{m}$, i.e. the group of the shortest interbands should represent less than 0.23 kb - 0.8 kb of DNA length. A comparable calculation was also done for the average band thickness. The average axial band thickness was calculated as being $0.21 \mu\text{m}$ with a standard deviation of $\pm 0.14 \mu\text{m}$. The relevance of these measurements in relationship to coding potentials and gene size will be considered in section 6.3.1. of the Discussion.

The average length of the SSP preparations analyzed of chromosome 6 and the standard deviation was $11 \pm 0.74 \mu\text{m}$. This is an approximate 2.2-fold degree of spreading in comparison with the $5 \pm 0.5 \mu\text{m}$ chromosome length measured in squash preparations (Berendes, 1963). The degree of spreading is less than those determined for longer chromosomes (see following sections). This could be a factor of both the strong heterochromatic nature of this chromosome and its short size in general.

5.3.2. Chromosome 4 in Drosophila hydei

(Source: based on II)

Pattern analysis: In Table 3 the banding pattern of chromosome 4 is listed as it was depicted in the original LM chromosome map based on squash preparations (Berendes, 1963). Although 394 bands can be seen in that map, Berendes (op. cit.) only claimed a total number of 388 bands. The difference probably lies in the two tight band groups in 86B and 91C which Berendes could have counted each as single polytene structures.

Berendes's (op. cit.) method of division and subdivision sectioning is somewhat different in comparison with the maps of other Drosophila species (e.g. Sorsa et al., 1984). If one follows exactly the sectioning given in Berendes's map, several divisions and subdivisions start in the middle of a doublet (e.g. 71B and 72B; see Table 3 and Figure 19). In the present study, Berendes's method of sectioning was used (for a review on the differences of chromosome sectioning in all the literature see Kalisch et al., 1985a).

The EM observations in the micrographs of ten SSP chromosome preparations showed a total number of 543 chromosome bands which is almost a 40% increase in comparison with the LM chromosome map. There are six subdivisions (71D, 76A, 83D, 85B, and 90C in which Berendes (op. cit.) found more bands than observed in the EM micrographs. In five of these cases, there is a difference of only one band, whereas subdivision 85B is a special case. In structures 85B1-2, which are always partly decondensed during the late third larval stage, only the number of bands were listed which could be clearly distinguished from each other.

In some electron micrographs, it appears that 85B1 and 85B2 are composed of several structures in close proximity, but there is still uncertainty about the total number of

them. Since no investigations on different developmental stages or different tissues have been carried out so far, only a preliminary number of bands can be given. The same situation is apparent in another thirteen subdivisions of chromosome 4 (76B, 78AB, 80A, 85A, 86A, 89AB, 90AB, 93A, and 93C). In the EM chromosome map of Figure 19, these polytene structures were plotted as "fields" (i.e. dotted or dashed areas; cf. with Kalisch et al., 1984).

In comparison with the original EM chromosome map of region 86A-94C (Reiling et al., 1984b), three additional bands were found: 87B3, 87D5, and 90D1. The left division border of 91A was also changed slightly to the right. Finally, there are some minor changes between both maps in 88A4, 88A6, 91C4, 91C6, and 91C7. If the sectioning of the present chromosome map in Figure 19 is compared with the EM photo map published in Kalisch et al. (1985a), there are minor differences in 73C, 74C-D, 80A-D, 81A-B, and 91A.

Quantitative analysis: Data from ten SSP chromosome preparations were used to calculate average data and to plot the chromosome map in Figure 19. The band types used (solid and dotted lines) represent subjective images of the chromosome bands as seen in the electron micrographs. Bands of equal thickness and band type, with interbands not greater than 0.094 μm (0.3 mm in the micrographs), have been listed as doublets and triplets in Table 3.

Chromosome outlines in Figure 19 are also based on measurements. However, this parameter shows a high variability in homologous SSP structures as was mentioned in Reiling et al. (1984b). In many of the SSP chromosome divisions of Figure 19 the outlines differ significantly from homologous divisions in the LM chromosome map, which is based on squash preparations (Berendes, 1963). The same is true if the EM photo map based on SSP chromosomes (Kalisch et al., 1985a) is compared with the LM photo map based on stretched and squashed chromosomes (Ananiev and

Barsky, 1982). Those differences may be caused, at least partially, by mechanical stretching on the one hand and by surface-spreading on the other. Additionally, it has been shown that the medium used for the excision of the salivary glands already has a strong influence on chromosome preparation (Kalisch and Whitmore, 1983).

In chromosome 4, type-1 constrictions can be found at 73C, 85BC, 89B, 90CD, and, with less prominence, also at 74D, 79AC, and 80C. Subdivisions 85BC and, less prominently, 90CD each carry one type-2 constriction.

For calculating the average interband length and average band thickness of chromosome 4, the "fields" (mentioned above: in subdivisions 76B, 78AB, 80A, 85A-C, 86A, 89AB, 90AB, and 93C), and the interbands between them had to be eliminated. For the rest of the chromosome (517 bands and 532 interbands) the average axial interband length was 0.33 μm . This represents a DNA length of between 0.97 kb and 3.3 kb. 0.29 μm was calculated for the average band thickness. The average value of band thickness is of interest in so far as the DNA material involved in an average band could be calculated. However, the average degree of DNA contraction within a band is not exactly known and may vary between bands or even within an individual band (Semeshin et al., 1985b). The relevance of the band/interband measurements in respect to coding potentials and gene length will be considered in section 6.3.1. of the Discussion.

The total length of chromosome 4 in Figure 19 is about 360 μm . This is about a 2.5-fold degree of longitudinal spreading in comparison with the average value found in squash preparations (Berendes, 1963).

5.3.3. X chromosome in Drosophila hydei

(Source: based on I)

Pattern analysis: In the original light microscopic (LM) map of the X chromosome (Berendes, 1963), several division and subdivision sectionings start in the middle of a doublet. This method of sectioning was followed in this present study. The LM based map (Berendes, op. cit.), also had to be revised as a prerequisite for the cytological measurements in the present study (Table 4 and Figure 20). Berendes reported 350 chromosome bands in the X, whereas one can count 391 in his hand-drawn LM chromosome map in the same paper (if one interprets all of the peculiarities of the pattern as individual bands). This fact was taken into consideration in Table 4 by giving two values for some subdivisions of the LM map (those in brackets could reflect Berendes's interpretation for a total of 350 bands).

526 chromosome bands were observed by electron microscopy (EM) using the technique of surface-spread polytene (SSP) chromosome preparation (about a 34% increase over the 391 bands mentioned above or a 50% increase over the 350 bands officially cited by Berendes (1963). This increase is lower compared with EM investigations of autosomal regions (Grond and Derksen, 1983; Whitmore et al., 1984; see section 5.2.1. of Results). On the other hand, comparable values were found in the EM analyses of chromosome 4 (see section 5.3.2. of Results).

In 7 out of the 81 subdivisions of the X chromosome (1A, 3C, 5C, 10A, 14D, 16A, and 18A; Table 4), the EM analysis showed fewer polytene structures than in Berendes's LM map. In four of these (1A, 5C, 10A, and 14D), the difference could simply be based on the interpretation of Berendes's hand-drawn chromosome map mentioned above, i.e. some of the peculiarities of the hand-drawn pattern do not represent individual bands (compare LM values in brackets of Table 4). In the remaining three subdivisions (3C, 16A, and 18A)

there is one chromosome band fewer per subdivision in the EM map. For the EM pattern of another four subdivisions (11C, 12B, 16D, and 20D) only preliminary results are given, because of the decondensed state of the bands in these subdivisions during the larval stage analyzed.

Quantitative analysis: Interband lengths, band thicknesses and band diameters of all the polytene structures of the X chromosome were measured individually in electron micrographs of ten SSP chromosome preparations. Chromosome bands and interbands with fuzzy borders and variations in size had to be measured several times and therefore, represent average values. Fuzzy borders are generally not a cytological indication of the pulling of band material into the interbands by the spreading technique used. In SSP chromosome preparations the total amount of chromosomal DNA is spread and extremely flattened which naturally leads to more superimpositions of this type than in thin sections of chromosome squash preparations (Sorsa, 1982b, c).

Furthermore, it has been shown by EM analyses that even the tiniest bands are not unravelled after strong spreading in SSP chromosomes (Kalisch and Jacob, 1983; Kalisch and Whitmore, 1983; Kalisch et al., 1985b). In contrast, one cannot exclude differences in the interband lengths as a result of different preparative techniques.

Based on the data from the ten SSP chromosome preparations analyzed, an EM map was plotted (for details see Methods section 4.2.) for the entire pattern of the X chromosome (Figure 20). Note that structures in those subdivisions with preliminary results (11C, 12B, 16D, and 20D) are depicted as dotted sections. Neighbored bands of the same band type and thickness with interbands not longer than 0.094 μm (i.e. 0.3 mm in the electron micrographs) were interpreted as doublets, triplets, or quadruplets in Table 4.

For the calculation of the average axial interband length, seven prominent interbands (in 11C, 12B, 16D, and 20D) were not included because of their neighboring decondensed polytene structures. The axial length of the remaining 518 interbands is approximately 194 μm . From this, the average axial interband length of the X chromosome is 0.38 μm . The standard deviation for these distribution frequencies is $\pm 0.25 \mu\text{m}$, i.e. ca. 2/3 of the 518 interbands are in the range of 0.13 μm to 0.63 μm . As a maximum length, 1.41 μm was measured for the right-hand interband of chromosome band 7D2.

If one accepts 0.34 $\mu\text{m}/\text{Kb}$ as a minimum and 0.10 $\mu\text{m}/\text{Kb}$ as a maximum DNA packing ratio in the interbands of SSP chromosomes, the average interband (0.38 μm) represents a DNA length between 1.1 Kb and 3.8 Kb. Due to the standard deviation of $\pm 0.25 \mu\text{m}$ mentioned above, ca. 1/6 of the interbands analyzed are on the average $\leq 0.13 \mu\text{m}$, i.e. the group of the shortest interbands should represent less than 0.38 Kb - 1.30 Kb of DNA length. A comparable calculation was also done for the average band thickness in the X chromosome. (For the same reason already mentioned above, 8 out of the 526 chromosome bands could not be used). The axial length of the remaining 518 bands was approximately 136 μm , thus, the average axial band thickness is 0.26 μm (standard deviation $\pm 0.14 \mu\text{m}$). The relevance of the band/interband measurements in relationship to coding potentials and gene size will be considered in section 6.3.1. of the Discussion.

The total length of the SSP chromosomes analyzed was $375 \pm 50 \mu\text{m}$, which may be compared with the $157 \pm 25 \mu\text{m}$ of chromosome squash preparations (Berendes, 1963). This 2.4-fold degree of spreading coincides with the 2.8-fold degree of spreading measured in *Drosophila virilis*, whereas a 3.6- to 5.4-fold degree was found in *D. melanogaster* (Kress et al., 1985). However, one has to consider that these degrees of spreading are based on data from squash preparations of

different investigations, i.e. the "well-extended" chromosomes measured could themselves be extended by different degrees and, therefore, may not be exactly comparable.

Nevertheless, an interband/band relationship can be calculated for SSP chromosomes by the total lengths given for interbands and bands, i.e. interbands (194 μm) : bands (136 μm) = 59% : 41%. Correspondingly, a 61% : 39% relationship (average interband length : average band thickness = 0.11 μm : 0.07 μm) has been found from measurements of chromosome squash preparations of Drosophila melanogaster (Sorsa et al., 1984). Even when these data are collected from different species of Drosophila, they support earlier measurements in Chironomus (unpublished data) which indicated that, on the average, interbands and bands are proportionally spread in SSP chromosomes in comparison with chromosome squash preparations.

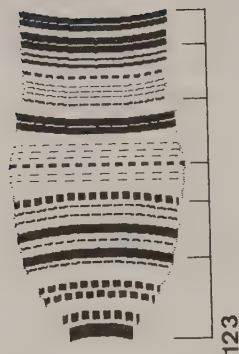


Figure 18. Computerized EM reference map of the band-interband pattern of salivary gland chromosome 6 in *Drosophila hydei*. Band thicknesses, band diameters, and interband lengths of the individual polytene structures depicted in this map are based on measurements in electron micrographs of six surface-spread polytene (SSP) chromosomes. The width shown is a 2:1 reduction due to the limitation of the plotter used. Division sectioning is according to the LM chromosome map of Berendes (1963). (Source: XIV)

Table 3 Banding pattern of salivary gland chromosome 4 in *Drosophila hydei*

Divisions and sub divisions	LM map t	EM map: D	S	t	%	Divisions and sub divisions	LM map t	EM map: D	S	t	%
71A	2	(11)	2	3		77A	3		4	4	
B	1	(1)	1	2		B	2		2	4	
C	4	1	5	3'		C	4	1	2	4	
D	4	1	1	3'		D	7	1+(1)	5	8	
71	12	2	9	13	8.3	77	17	2+(1)	15	20	17.6
Totals						Totals					
72A	3	1+(1)	1	4		78A	2	(1)	1	2*	
B	3	(1)	1	4		B	4	1	6	6*	
C	4	1	5	5		C	4	1	4	6	
D	3	1	2	4		D	8	1	8	11	
72	13	3	12	18	38.5	78	18	1+(1)	19	25	38.9
Totals						Totals					
73A	3	2	1	5		79A	6	(1)	9	10	
B	5	1	6	8		B	3	(1)	3	4	
C	4	1	4	4		C	5		10	10	
D	3	1+(1)	4	6		D	3	1	3	3	
73	15	4+(1)	14	23	53.3	79	17	1	25	27	58.8
Totals						Totals					
74A	2	(1)	1	2		80A	4		6	6*	
B	3	1	3	5		B	4	1	5	5	
C	5	1+(1)	2	5		C	5	1	5	5	
D	5	3+(2)	9	17	13.3	D	2	1	4	4	
74	15	3+(2)	9	17	13.3	80	15	1	21	23	53.3
Totals						Totals					
75A	4	(2)	3	5		81A	2		4	4	
B	4	(1)	4	5		B	3	1	3	11	
C	5	1	6	6		C	6	1	9	3	
D	5	1	6	6		D	3	1	2	2	
75	19	1+(2)	18	22	15.8	81	13	1	18	20	53.8
Totals						Totals					
76A	5	(1)	3	4*		82A	4		7	7	
B	5	1	5	5*		B	7	1	8	8	
C	4	1	6	6		C	5	1	6	8	
D	4	1	6	8		D	3	1	4	4	
76	18	1+(1)	20	23	27.7	82	19	1	25	27	42.1
Totals						Totals					

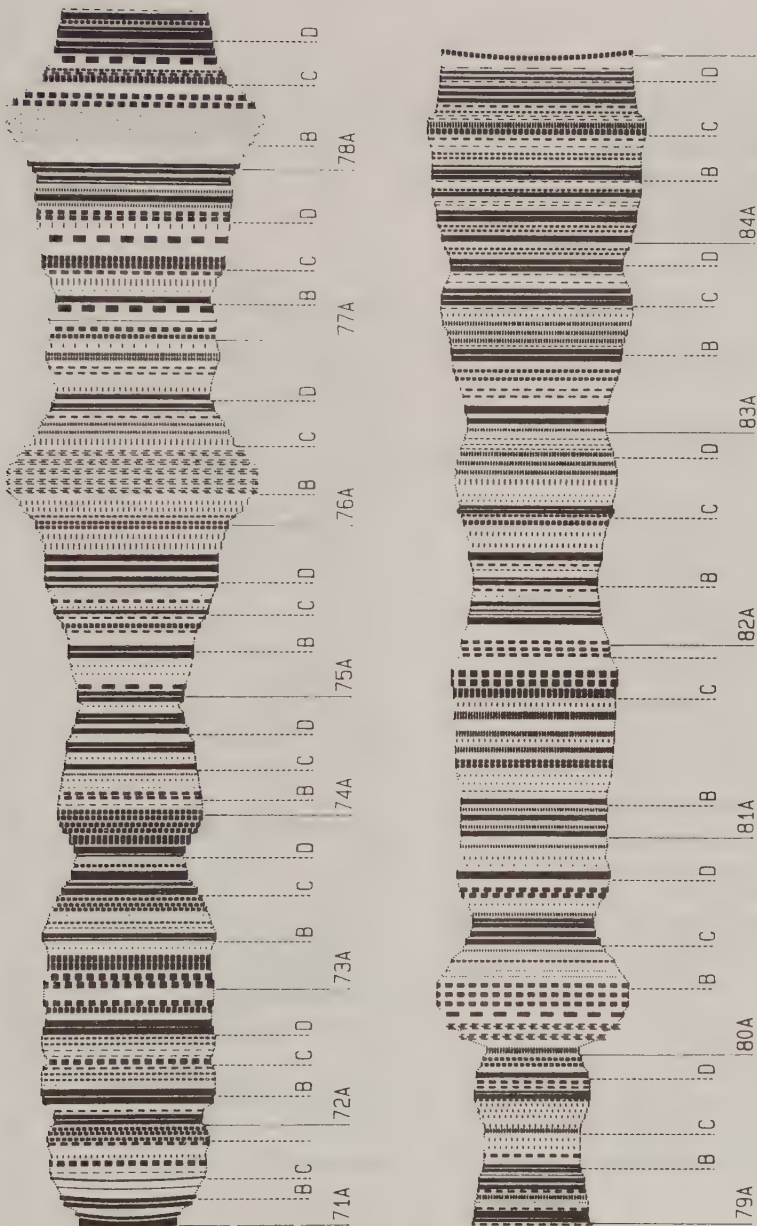


Figure 19. Computerized reference map of the band-interband pattern of salivary gland chromosome 4 in *Drosophila hydei*. Dotted 'fields' in 76B, 78AB, 80A, 85AC, 86A, 89AB, 90AB, 93A, and 93C represent a preliminary pattern (see text). Sectioning of divisions and subdivisions coincides with the original LM chromosome map (Berendes, 1963). Bar equals 10 μ m in SSP chromosomes. (Source: II)

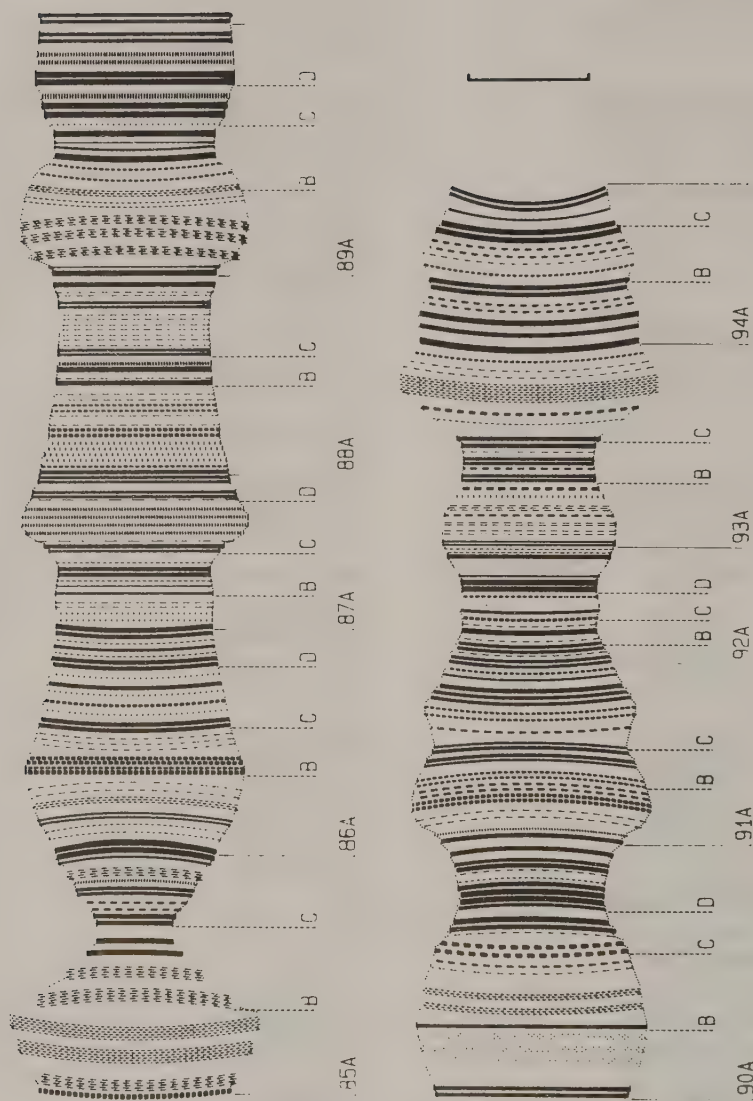


Figure 19 (continued)

Table 4. Banding pattern of the salivary gland X chromosome in *Drosophila hydei*. Comparison between the LM chromosome map (Berendes 1963) and the EM chromosome map of Figure 20.

Divisions and subdivisions	LM map		EM map			
	t	T	D	S	t	%
1A	14 [10]	—	1	9	11	^b
B	8 [7]	—	—	11	11	
C	3	—	1	4	6	
D	4	—	—	5	5	
1 Totals	29	—	2	29	33	13.8
2A	7	1Q	—	4	8	
B	5	—	1	5	7	
C	3	—	1	3	5	
D	4	—	1	2	4	
2 Totals	19	1Q	3	14	24	26.3
3A	5	—	1	5	7	
B	4	—	1	4	6	
C	6	(1)	1	2	5	^b
D	5 [4]	(1)	—	5	7	
3 Totals	20	1	3	16	25	25.0
4A	6 [5]	1	—	4	7	
B	4	1	—	3	6	
C	4	—	—	5	5	
D	5	—	—	6	6	
4 Totals	19	2	—	18	24	26.3
5A	3 [2]	—	(1)	3	4	
B	3 [2]	—	(1)	2	3	
C	7 [6]	—	1	4	6	^b
D	3	1	1	1	6	
5 Totals	16	1	3	10	19	18.8
6A	5	—	1	6	8	
B	4 [2]	—	—	5	5	
C	7 [6]	—	—	11	11	
D	3 [2]	—	—	3	3	
6 Totals	19	—	1	25	27	42.1
7A	4	—	1	4	6	
B	4 [3]	—	—	5	5	
C	3 [2]	—	(1)	3	4	
D	6 [4]	—	(1)	5	6	
7 Totals	17	—	2	17	21	23.5
8A	4	—	—	4	4	
B	3	—	—	5	5	
C	4 [3]	—	—	6	6	
D	2	—	—	4	4	
8 Totals	13	—	—	19	19	46.2
9A	4	—	1	3	5	
B	5	—	1	4	6	
C	5	—	1	3	5	
D	4	—	—	6	6	
9 Totals	18	—	3	16	22	22.2

Table 4 (continued)

Divisions and subdivisions	LM map t	EM map			t	%
		T	D	S		
10A	7 [5]	—	—	6	6	^b
B	2	—	—	4	4	
C	4 [2]	—	—	10	10	
D	5	1	—	2	5	
E	5 [4]	—	—	7	7	
10 Totals	23	1	—	29	32	39.1
11A	9 [7]	—	2+(1)	7	12	
B	7	1	2+(1)	5	13	
C	6	1	1+(1)	5	11	^a
D	4 [3]	—	1+(1)	2	5	
11 Totals	26	2	8	19	41	57.7
12A	6 [5]	—	1	4	6	
B	5	—	(1)	10	11	^a
C	3	—	(1)	9	10	
D	6 [5]	—	1	9	11	
12 Totals	20	—	3	32	38	90.0
13A	5 [4]	—	—	6	6	
B	5	—	1	4	6	
C	6	—	(1)	6	7	
D	3	—	(1)	4	5	
13 Totals	19	—	2	20	24	26.3
14A	4	—	2	2	6	
B	5	—	2	3	7	
C	3 [2]	—	—	3	3	
D	6 [4]	1	—	2	5	^b
14 Totals	18	1	4	10	21	16.7
15A	5	—	1+(1)	6	9	
B	4	—	(1)	4	5	
C	3	—	2	1	5	
D	4	—	—	4	4	
15 Totals	16	—	4	15	23	43.8
16A	5	—	—	4	4	^b
B	4	—	—	4	4	
C	2	—	—	3	3	
D	4	—	—	6	6	^a
16 Totals	15	—	—	17	17	13.3
17A	5	—	1	4	6	
B	6 [5]	—	—	8	8	
C	4 [2]	—	1	2	4	
D	6	—	1	8	10	
17 Totals	21	—	3	22	28	33.3
18A	3	—	—	2	2	^b
B	5 [3]	—	—	5	5	
C	7 [5]	—	2	5	9	
D	6	—	1	7	9	
18 Totals	21	—	3	19	25	19.0

Table 4 (continued)

Divisions and subdivisions	LM map t	EM map				t	%
		T	D	S			
19A	3	—	—	3		3	
B	4	—	—	7		7	
C	6	—	1	7		9	
D	8 [7]	—	1	9		11	
19 Totals	21	—	2	26		30	42.9
20A	4	—	2	1		5	
B	5	1	1	8		13	
C	4	—	1	5		7	
D	8	—	—	8		8	^a
20 Totals	21	1	4	22		33	57.1
1-20 Totals	391 [350]	1Q + 9.50		395		526	34.5 50.3

t total number of bands (for the LM map values in brackets see text). Q quadruplet in 2A. T number of triplets, D number of doublets (T and D values in parentheses indicate that individual bands belong to neighbored divisions or subdivisions according to Berendes' sectioning method). S number of single bands

^a subdivisions of the EM map with a preliminary number of bands, see text, ^b subdivisions of the EM map with fewer chromosome bands in comparison with the LM map. % percentage increase in additional bands in the EM chromosome map

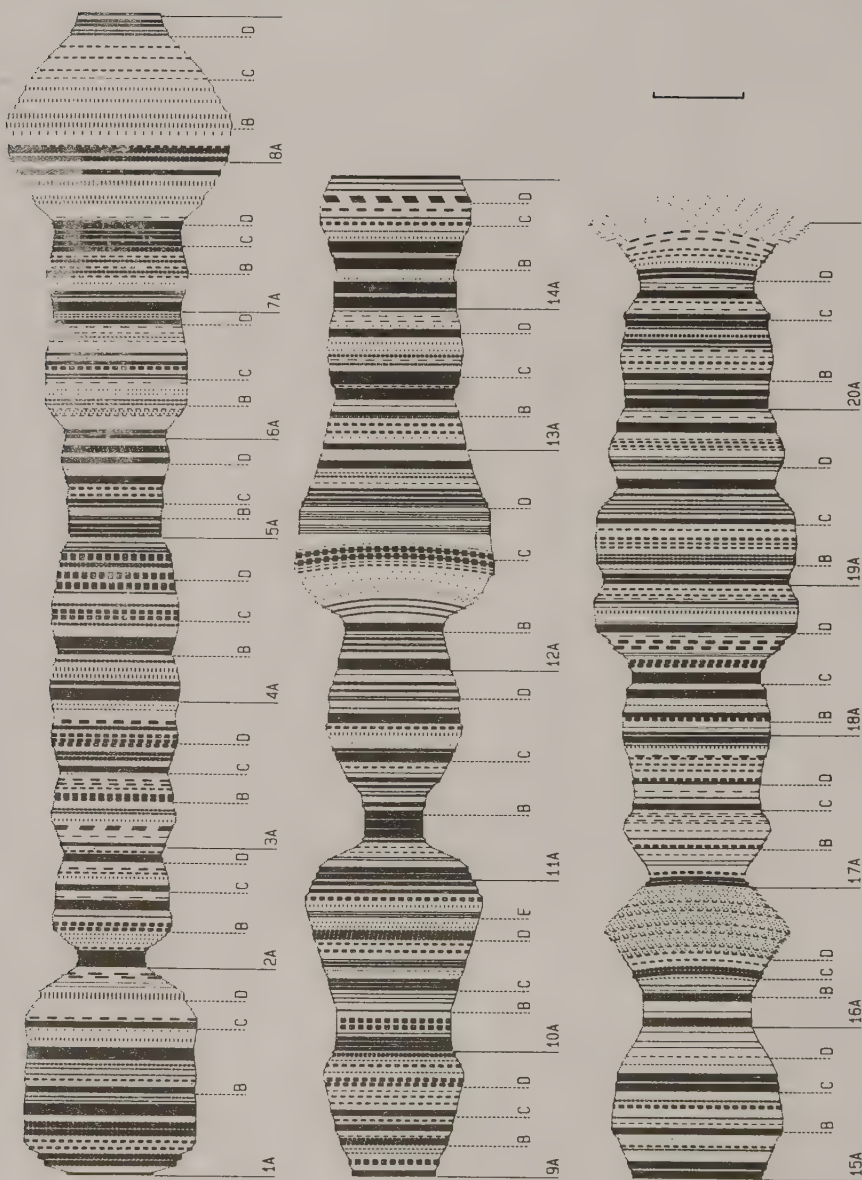


Figure 20. Computerized reference map of the band-interband pattern of the salivary gland X chromosome in *Drosophila hydei*. Band thicknesses, band diameters, and interband lengths of individual polytene structures depicted in this map are average values calculated from measurements in electron micrographs of ten SSP chromosome preparations. Sectioning of divisions and subdivisions coincides with the original LM chromosome map (Rendones, 1963). Bar represents 10 μ m. (Source: I)

5.4. Comparative EM photo maps of salivary gland and Malpighian tubule chromosomes from Chironomus thummi piger (Source: Whitmore, unpublished)

The electron microscopic photo maps of the salivary gland and Malpighian tubule chromosomes of Chironomus thummi piger depicted in this section represent the first comparative EM investigation on polytene chromosomes from different tissues which is not just for a single chromosome, but for an entire genome.

In addition to the EM photo maps, intended for general mapping purposes, specific comparisons are made of:

- puffing patterns,
- banding patterns, chromosome lengths and widths,
- physical appearance of intercalary heterochromatin, and
- telomeres.

The validity of LM observations by various authors on the constancy and variability in chromosomal banding patterns is discussed on hand of the comparative EM results.

5.4.1. Earlier comparative studies on polytene chromosomes in different tissues

In general, comparative studies of the banding and puffing patterns between polytene chromosomes originating from different tissues of an individual species may well provide an important facet in a better understanding of the relationship between the structure and function of polytene chromosomes. Relatively few comprehensive investigations have been undertaken, however, in either category. This has been primarily due to simply the lack of tissues exhibiting as highly polyploid nuclei as found commonly in the salivary

glands of many dipteran and also, the resulting limited resolution of those chromosomes in light microscopy.

Concerning the constancy of the banding pattern

From a historic perspective, the question as to the constancy of the banding pattern between chromosomes of different tissues has been a controversial topic. In 1940, Berger found, in a comparison of salivary gland and midgut polytene chromosomes of Sciara ocellaris, that the banding patterns were similar. Kosswig and Sengün (1947), Sengün and Kosswig (1948), and Pennypacker (1950), in examining polytene chromosomes in a number of Chironomus tissues, including salivary gland, midgut, Malpighian tubule, and anal gill, came to the contrary conclusion that the banding patterns were not constant between chromosomes of different tissues. Sengün (1954) also stressed the variability in the chromosomal banding pattern between different cells of the same tissue in Drosophila repleta. Slizynski (1950) working with Drosophila melanogaster, Beermann (1950; 1952) with Chironomus tentans, as well as Pavan and Breuer (1952) with Rhynchosciara angelae, reconfirmed the original conclusion of Berger (op. cit.) that the banding patterns between polytene chromosomes of different tissues are basically constant with most variations being due to tissue specific puffing.

Specifically concerning the constancy of the banding pattern, Beermann (1972) came to the following conclusions based on his light microscopic comparison of the third chromosome from four different tissues of Chironomus tentans (Beermann, 1952):

"subtle tissue-specific variations in the microscopic appearance of single bands, or small groups of bands...is rather small and in many cases due to differential puffing...the general characteristics of banding, such as number of bands per unit length

as well as the specific banding sequence of homologous chromosome sections, are nearly identical."

Beermann (op. cit.) classified tissue-specific variations which were probably not due to differential puffing as following:

Presence-absence: "A specific band that is seen in one or several different tissues cannot be demonstrated in another tissue. This has only been the case of very thin bands."

Singlet-doublet: "A band may appear double in one tissue and always as a unit in another."

Differential spacing: "The distance relationships...of bands...appear specifically changed in one tissue as compared to the others..."

Tissue specific increased staining of the interbands...

Differences in the intensity of staining of bands...

Beermann (op. cit.) made then an important generalization which most of the studies to follow usually supported: "The number and sequence of bands and interbands are constant in all tested somatic tissues...A given band or interband may look specifically different in different cell types, but nevertheless it seems to represent the same individual sub-unit or subsection of the genome." One must note, however, the relatively large differences in the band numbers observed by Beermann (op. cit.) in this fundamental work (i.e. salivary glands - 380 bands; Malpighian tubules - 440 bands; rectum - 380 bands; midgut - 490 bands).

Some 30 years later to the original studies of Beermann (1950; 1952), Ribbert (1979), in comparing experimentally

induced polytene chromosomes in Calliphora erythrocephala, was not able to homologize the banding patterns between the highly polyploid germ-line nurse cells (NC) and the pupa bristle forming cells (trichogen cells (TC)). Using the same approach, Ribbert (personal communication) has reached the same conclusion working with two other species. Although Zhimulev et al. (1981) believes to have seen some homology in the chromosomes depicted by Ribbert (1979), the reason why the banding patterns should show such differences is still open to speculation.

Redfern (1981a) reinvestigated, therefore, the question of the banding pattern homology in Anopheles stephensi where naturally-occurring polytene chromosomes are found in the nurse cells. He concluded that the banding patterns of the salivary gland and ovarian nurse cell chromosomes were essentially similar. Most recently, both Sihna et al. (1987) and Heino (1989) have made comparisons of polytene chromosomes from the salivary glands and pseudonurse cells (PNC) of the Drosophila melanogaster "otu" mutant. They found the banding pattern of the chromosome(s) examined to be very similar.

Variations have been observed, however, in the banding patterns of salivary gland chromosomes themselves. In Chironomus plumosus, for example, changes in chromosome lengths and the number of observable bands can be seen in relationship to seasonal changes in temperature (Ilinskaya and Maximova, 1976; Ilinskaya and Maximova, 1978) and physiological differences in the environmental conditions such as the salt concentration (Belyanina, 1977).

Zhimulev and coworkers proposed a model of dynamic organization of polytene chromosomes (reviewed in Zhimulev et al., 1981) based primarily on their observations of the transcriptional activity and band variation in Drosophila melanogaster. The essential points of their model are:

(1) "At all levels of chromosome organization, the process of chromatin decondensation correlates, in one way or another, with the transcriptional activity...DNP condensation (a compact band, and the puff formed from it), reflect the minimum and maximum transcriptional levels respectively. The interbands whose material seems to be decondensed in the same degree as the puff material should also be considered as minor puffs..."

(2) "The banding patterns are only relatively constant, i.e., in normally functioning cells with a background of considerable stability, one can find some cases of variation: elongation of the interbands and splitting of the bands..."

(3) "The number of essential loci in...chromosomal regions...corresponds to (or is somewhat larger than) the number of bands/interbands in the region..."

(4) "It seems reasonable to distinguish two types of genes...those which function permanently and provide for the general cellular functions...and the tissue- and stage-specific genes."

Zhimulev et al. (op. cit.) suggest that the overall banding pattern is more or less a result of the activity of so-called "housekeeping" genes located in the interbands and that the similarity of the banding pattern between chromosomes of different tissues is merely a reflection of the similarities in cellular requirements.

Concerning puffing patterns in different tissues

Comparative studies of the patterns of puffing activity in polytene chromosomes of different tissues in various dipteran species have been completely limited to the light microscopic level (e.g. Berendes, 1966; Holden and

Ashburner, 1978; Redfern, 1981b; Gupta and Singh, 1983; Singh and Gupta, 1985; Staiber and Behnke, 1985; Hochstrasser, 1987; Roberts, 1988). Here, one can say that there is a general consensus that between chromosomes of different tissues there are puffs which are common, some which are tissue specific, and some which are stage specific.

For example, Berendes (1966) found in Drosophila hydei, three tissue specific puffs by the Malpighian tubules, four by the midgut, and seven by the salivary glands. Gupta and Singh (1983) reported by Melanagromyza obtusa of five tissue specific puffs by the salivary glands, twelve by the midgut, and twelve common puffs. And Staiber and Behnke (1985) found in Acricotopus lucidus that the Malpighian tubules have nine tissue specific puffs compared with the salivary glands. In comparison with most Drosophila species, the Chironomus thummi subspecies, for which there have been no LM or EM comparative investigations of tissues other than the salivary glands so far in the literature, display relatively few prominent puffs. It would be of interest, therefore, to also study this factor in different tissues.

In Beermann's opinion (1956; 1972), in order to definitely resolve questions associated with the chromosomal banding patterns necessitates the use of electron microscopy. There have been, however, virtually no enddepth electron microscopic studies of polytene chromosomes published from tissues other than those of the salivary glands with the following exceptions:

Electron micrographs of unidentified Drosophila melanogaster fat body chromosomes were published by Ris (1976) and Martin and Sedat (1982). Laird was able to identify a short section of a fat body from D. melanogaster. Ten Tusscher and Derksen (1982), using the squash and thin-sectioning technique, presented EM micrographs of the

fourth chromosome of Chironomus tentans Malpighian tubules. Kalisch and Hägele (1982) published for demonstration purposes, electron micrographs of unidentified Malpighian tubule chromosome sections from Chironomus tepperi.

Concerning intercalary heterochromatin

Kaufmann (1939), first used the term "intercalary heterochromatin" to denote regions other than the telomeric and centromeric heterochromatin which display "ectopic pairing" or other types of chromosomal aberrations.

Up to now, enddepth cytogenetic investigations of intercalary heterochromatin have been based primarily on Drosophila chromosomes (reviewed in Beermann, 1962; Gvozdev, 1981; Zhimulev et al., 1982; see also Boshakov et al., 1985; Lamb and Laird, 1987; Kholodilov et al., 1988). Since the genetic nature of intercalary heterochromatin still remains mostly undetermined, one is restricted to describing the characteristics which these regions exhibit such as:

- ectopic pairing between sections of the same chromosome or between different chromosomes,
- late replication and local underreplication,
- weak points and constrictions,
- the presence of inverted repeats.

Zhimulev et al. (1982) differentiate the morphology of weak points into the following categories: (1) constrictions, (2) breaks, (3) semibreaks, and (4) shifts (offsets). Furthermore, the morphology of ectopic contacts can also be divided into two major types: (1) ectopic pairing in which nonadjacent bands are connected by compact DNP material. This type can be usually detected using light microscopy. And (2) ectopic pairing between two fairly adjacent bands. This type can only be detected with confi-

dence using electron microscopy (Zhimulev et al., op. cit.).

Intertissue differences in the appearance of intercalary heterochromatin have been noted in the following cases:

Pavan and Breuer (1952) found by Rhynchosciara angelae that the heterochromatin was more differentiated in the Malpighian tubule chromosomes than in the salivary gland chromosomes. They stated that "in the salivary glands, the heterochromatin very often appears to be rather similar to the euchromatin." They also observed that while the heterochromatin by the Malpighian tubules and vesiculae seminales tended to form a chromocenter, the salivary glands did not.

Ribbert (1979) reported by the comparison of experimentally induced highly polyploid germ-line nurse cells (NC) chromosomes with those of the pupal bristle forming cells (trichogen cells (TC)) by Calliphora erythrocephala, that the TC-polytene chromosomes showed extensive ectopic pairing, break points and constrictions, whereas the higher polytenized NC chromosomes did not. Ribbert concluded that "The comparative cytology of TC- and NC-polytene chromosomes strongly supports the suspicion that local underreplication might occur in somatic polytene chromosomes, e.g. from trichogen cells, salivary gland cells, cells of Malpighian tubules etc., but not in the secondary polytene chromosomes of the abortive oocytes."

Roberts and MacPhail (1980) found, on the one hand, in comparing salivary gland and fat body chromosomes in Drosophila gibberosa, that "Midway in the euchromatic arm of acrocentric chromosome 2...is a region of frequent breaks in squashed salivary gland chromosomes. Polytene chromatin at this point appears con-

sistently narrowed...in the salivary chromosome 2. The chromosome 2 of the fat body, however, has no tendency to break in this region; instead, the fat body chromosome 2 has in this region three broad, dark bands. The marked narrowing of the salivary chromosome is at this point and is of such a degree that the bands cannot be seen. It appeared, therefore, that the differentiation of somatic cells can include differential replication of a few of the chromomeres of a polytene chromosome." Roberts (1988) also observed by Drosophila gibberosa, on the other hand, that the bands in division 81E of chromosome 5L were more condensed by the midgut chromosomes forming a constriction not seen in the salivary gland, fat body or Malpighian tubule chromosomes.

In a comparison of salivary gland and hindgut chromosomes in Drosophila melanogaster, Zhimulev et al. (1982) observed that there were great differences in the relative thickness of the bands which formed weak points in the two tissues. In the lower polytenized hindgut chromosomes these bands appeared relatively thicker than in the salivary gland chromosomes. They also found the break frequencies to be much lower in the hindgut chromosomes. As a possible explanation they hypothesize that "It is conceivable that after each round of replication the relative number of underreplicated chromatids...is increased, which could cause increased breakability of the chromosomes..."

Finally, Heino (1989) in comparing polytene chromosomes from salivary glands and ovarian pseudonurse cells of the Drosophila melanogaster "otu" mutant, did not find constrictions or breaks in the PNC chromosomes, even at sites homologous to notably underreplicated sections in the salivary gland chromosomes. This he believes to indicate "that these sites...are

equally replicated along with the rest of the chromosomes in the PNC nuclei."

5.4.2. The salivary gland chromosomes of Chironomus thummi piger

Chironomus thummi piger is the phylogenetically older member of the chironomid subspecies Chironomus thummi thummi and Ch. thummi piger (Keyl, 1962; Schmidt et al., 1982). Chromosomes from the salivary glands of these chironomids have been used extensively for cyto-genetical investigations concerning not only chromosomal evolution, but also the structural organization of various chromosomal constituents (e.g. Keyl, 1962; Hägele, 1970; 1977; 1984; 1985; Kurth et al., 1978; Kurth and Bustin, 1981; Schmidt et al., 1980; Schmidt, 1981; Schmidt and Godwin, 1983). The evolutionary divergence of the two subspecies has resulted in a number of structural changes seen in the centromeric regions of the chromosomes as detailed by Keyl (1957) in hand-drawn maps of these sections in Chironomus th. thummi x Ch. th. piger hybrids. These differences include variations in band numbers, spacing, and degree of heterochromatization of individual polytene structures in homologous chromosome sections. The Ch. th. piger genome has also 30% less DNA in its genome than Ch. th. thummi as a result of DNA duplications of individual bands in Ch. th. thummi chromosomes (Keyl, 1965). Complete, hand-drawn chromosome maps exist only for Chironomus thummi thummi (Bauer, 1935; Keyl, 1957; Hägele, 1970).

Electron micrographs of the four salivary gland polytene chromosomes of Ch. th. piger are shown in Figures 23 - 26. In accordance with the published maps of Ch. th. thummi (Keyl, 1957; Hägele, 1970) the right chromosome arms are pictured on the left-hand side. The chromosomes were subdivided according to the comparative chromosome maps of Ch. th. thummi and Ch. th. piger of Keyl (op. cit.). Hägele (op. cit.) further subdivided these divisions in his

revised map of Ch. th. thummi. Due to the structural differentiation of the two subspecies mentioned above, however, this further subdivision is not directly applicable to the centromeric divisions of the Ch. th. piger chromosomes in its present form. For that reason, it has not been used except in the detailed presentations of areas where there is an exact homology between Ch. th. th. and Ch. th. pi. (Figures 21, 22, and 32).

It should be emphasized here that the determination of the banding patterns in the central divisions of Ch. th. piger is not as easy a task as it is for Ch. th. thummi which has, in comparison, rather large and quite distinctive centromeric regions. Several chromosome preparations were examined, therefore, to determine the exact borders of the divisions.

For the EM photo maps in Figures 23 - 26, SSP chromosomes were purposely chosen with a moderate degree of spreading, because these chromosomes still bear a strong resemblance in their contour and general appearance to squash preparations. This simplifies subdividing and mapping the divisions when making the initial comparisons between light microscopic squash preparations and electron microscopic SSP chromosome preparations. The chromosomes depicted have a ca. 2.0 - 2.4 fold longitudinal and 2.4 - 3.4 fold lateral degree of spreading in comparison to good squash preparations from chromosomes of comparable polyteny. Areas of special interest, for instance, chromosome sections with tight constrictions, can be more easily analyzed on chromosomes with a higher degree of spreading (e.g. the constriction in chromosome II at A2 seen in Fig. 24, at a lower-, and in Fig. 21, at a higher degree of spreading). Well spread Chironomus th. piger chromosomes can reach more than a ca. 3 fold longitudinal and 5 fold lateral enlargement over routine squash preparations as demonstrated in Figure 22 for a short section of chromosome II.

The general LM cytological characteristics of the salivary gland chromosomes of Chironomus th. thummi, as well as the major differences between the subspecies Ch. th. piger and Ch. th. thummi, have been described in detail elsewhere (Bauer, 1935; Keyl, 1957; Hägele, 1975). Therefore, just a few of the LM prominent features will be mentioned which are still evident in electron microscopic SSP preparations of the four chromosomes and help for an easy identification of the chromosomes:

Chromosome I of Ch. th. piger:

The salivary gland chromosome I (Figure 23) can be easily recognized from its flaired bell shaped end of the right arm which narrows into a tight constriction at A4/B1. This constriction is bordered by three thick double bands on the A4 side and two thick bands in B1. The centromeric region is submedian (division D).

Chromosome I is also the longest of the four chromosomes followed by II, III, and IV in that order and at the ratio of ca. 1 : 0.97 : 0.63 : 0.26 for the SSP chromosomes depicted in Figures 23 - 26, in comparison to ca. 1 : 0.9 : 0.7 : 0.25 found by Keyl (1957) for squash preparations of Ch. th. thummi. The chromosome I shown in Figure 23 has a length of 479 μm and an average width of ca. 33 μm .

Chromosome II of Ch. th. piger:

The salivary gland chromosome II (Figure 24) has a prominent constriction on the right arm in division A2 with two double bands at the narrowest part. The centromeric region is median (division C). The chromosome depicted has a length of 467 μm and an average width of ca. 25 μm .

Chromosome III of Ch. th. piger:

The salivary gland chromosome III (Figure 25) is noticeable by its lack of constrictions typical for chromosomes I and II. The right arm can be determined by the two thick bands at the beginning of division A2 and the appearance of a large diffused band in this section. The centromeric region is submedian (division B). In both LM squash preparations and EM SSP preparations, the right chromosome arm is wider than the left. The chromosome depicted has a length of 300 μ m and average widths of ca. 25 μ m for the right arm and 21 μ m for the left arm.

Chromosome IV of Ch. th. piger:

The salivary gland chromosome IV (Figure 26) has three large Balbiani rings in divisions B, C, and E. Division D carries the nucleolus. The heaviest concentration of thick bands in this chromosome can be seen on the right arm. The end of the left arm is characteristically funnel shaped. The centromeric region is terminal (division E). The chromosome depicted has a length of 126 μ m.

The micrographs of the salivary gland chromosomes depicted in Figures 23 - 26 are shown here on an extremely reduced scale to those which were used for the actual mapping. With a final magnification of 3,200 x from 1,600 x negatives, chromosome II in Figure 24 had, for example, a length of ca. 1.45 meters and a width of ca. 6 - 10 cm. Figure 22 shows an example of the normal enlargement size used. Such enlargements enable the measurement of bands and interbands for use, for instance, in computer based studies of the banding pattern as shown in section 5.3. for Drosophila chromosomes.

5.4.3. The Malpighian tubule chromosomes of Ch. th. piger

Up to now, there have been no investigations, in any form, carried out involving the Malpighian tubule chromosomes of Chironomus thummi piger. According to Beermann (1952), however, the chironomid designated by Sengün and Kossig (1947) as "Chironomus 3" is most likely the subspecies Ch. th. thummi.

Electron microscopic photo maps of the four Malpighian tubule chromosomes of Chironomus thummi piger are shown in Figures 27 - 30. The chromosomes were subdivided using the identical divisioning as in the salivary gland chromosomes. The Malpighian tubule chromosomes were compared to those of the salivary glands in following specific aspects:

- (A) Puffing patterns,
- (B) Banding patterns, chromosome lengths and widths,
- (C) Physical appearance of intercalary heterochromation,
- (D) Telomeres.

(A) Puffing patterns

Relatively rare in the salivary gland chromosomes I, II, and III are regions of transcriptional activity as signaled by the appearance of prominently puffed areas. There are only a few sections which show the typically large puffs evident in most Drosophila species. Some of the noticeable exceptions are seen, for example, in late fourth instar larvae, in division G1 of chromosome I or in divisions A3 and B4 of chromosome II. The majority of the transcriptionally active sites are of a rather discreet nature and noticeable, if at all, through a slightly diffused and granular appearance of the chromatin material. By ³H-uridine incorporation, which indicates the level of RNA synthesis, 336 transcriptionally active sites were determined,

however, in Ch. th. thummi salivary gland chromosomes (Kiknadze, 1981). Similar values have been found with 227 in Ch. tentans (Pelling, 1964), 341 - 355 in Drosophila melanogaster (Zhimulev, 1974; Vlassova et al., 1985), and 349 in Acricotopus lucidus (Staiber and Behnke, 1985). Further investigations of this type have not been undertaken with either Drosophila chromosomes or with Chironomus chromosomes of tissues other than the salivary glands.

This present study is limited to the determination of possibly transcriptionally active sites by band decondensation in the Malpighian tubule chromosomes and a cross-checking of these sites with the respective salivary gland chromosomes and also with those determined by Kiknadze (op. cit.) for the salivary gland chromosomes of Ch. th. thummi.

The puffing patterns of the Malpighian tubule chromosomes are of interest in contrast to those of the salivary glands in that not only were all major visible salivary gland chromosome puffs evident in the Malpighian tubule chromosomes, but also many of the transcriptionally active sites first seen through ^3H -uridine incorporation in the salivary gland chromosomes in Ch. th. thummi (Kiknadze, op. cit.) were present as large distinctive puffs in the Malpighian tubule chromosomes, e.g. in divisions A1 and E2 of chromosome II and in division B3 of chromosome III. A total of 71 common and possibly transcriptionally active sites were determined by band decondensation in the Malpighian tubule chromosomes. ^3H -uridine incorporation experiments have not been carried out as of yet. The EM observations apparently indicate, that there may be a correlation not only between the physical appearance of a puffed region and the transcriptional activity of that region, but also to the level of polyteny in the tissue being studied. This is an entirely new aspect which has not yet been discussed anywhere in the literature.

The Malpighian tubule chromosomes also exhibited a total of 34 tissue specific puffed regions; 12 on chromosome I, 11 on chromosome II, 7 on chromosome III, and 4 on chromosome IV. The higher number found here than detected in comparable light microscopic studies (e.g. 3 - 7 by Drosophila hydei (Berendes, 1966); 5 - 12 by Melanagromyza obtusa (Gupta and Singh, 1983); 9 by Acricotopus lucidus (Staiber and Behnke, 1985)), can be most likely attributed to the better resolution of the Malpighian tubule chromosomes using electron microscopy together with chromosomes prepared by the SSP chromosome technique.

It is of interest to note that in contrast to Chironomus tentans, where "there is no puffing in Malpighian tubules, rectum, and midgut chromosomes at those loci which form Balbiani-rings in the salivary glands" (Beermann, 1956), the Malpighian tubule chromosome IV of Chironomus th. piger clearly shows large puffs at positions equivalent to those of the Balbiani-rings in divisions B and C of the Ch. th. piger salivary gland chromosome IV.

Probably, the best studied Balbiani-rings are BR2 and BR1 of Ch. tentans, where 75S RNA molecules are synthesized and translated into large secretory proteins (reviewed in Sass, 1984). Even though experimental data does not exist concerning these puffs by Ch. th. piger, I assume a similar function of these areas of the fourth chromosome in the Malpighian tubules and salivary glands. This does not necessarily imply or rule out that the same protein(s) are being coded for in both tissues since there is a great number of bands involved in each of the puffed regions. This is a point which would be of future interest to investigate further.

Listed in Tables 5 - 8 are the respective divisions where puffs occur which are tissue specific for the Malpighian tubule chromosomes and those which have puffs common to both the Malpighian tubule and salivary gland chromosomes.

An example of comparative electron micrographs of the puffing patterns from the salivary gland and Malpighian tubule chromosomes in Chironomus thummi piger:

The electron micrographs in Figure 31 depict a side by side comparison from divisions F4d - G2l of chromosome I of the two tissues in which the puffing patterns and general banding patterns are clearly identical in both tissues. Each show a granular/diffuse type puff at G1e and a prominent puff at G1i. Concerning the banding pattern, however, subdivisions F4j and F4l each contain a very fine, modulating band structure. This structure appears in different preparations either as a diffuse, but tightly synapsed double band or as three extremely fine single bands.

From the cytological point, these are examples of band splitting as proposed by Zhimulev et al. (1981). Interestingly, in comparing the hand-drawn chromosome maps of Ch. th. thummi, Hägele (1970) depicted the homologous structures in F4l as two dotted bands, while Keyl (1957) drew three fine single bands, for a total of three or four bands, respectively, for this subdivision. Of the 74 bands shown in Hägele's revised map of this section (when one includes all interpretable structural peculiarities as bands) or 75 in Keyl's map, only one band in subdivision F4h could not be found in the EM micrographs of either the salivary glands or the Malpighian tubule chromosomes. The missing band may be in this case a stage specific mini-puff.

(B) Banding patterns, chromosome lengths and widths

An example of comparative electron micrographs of the banding patterns from the salivary gland and Malpighian tubule chromosomes in Chironomus thummi piger:

Figure 32 is a side by side EM comparison of divisions C3 - E1 of chromosome I of the salivary glands and the Malpighian tubules. The chromosomes were isolated from a single larva and both of the sections are depicted here using the same enlargement factor. The subdividing of the chromosomes into the groups 13 - 33 is according to the hand-drawn map of this section by Keyl (1957), which was based on LM analyses of salivary gland chromosomes from squash preparations of Ch. th. th. x Ch. th. pi. hybrids.

The banding patterns of the SSP salivary gland and Malpighian tubule chromosome preparations differ from the LM chromosome map from Keyl (op. cit.) primarily in the following subdivisional groups: C3 group 13 - an additional band can be seen before the first band drawn in this group on the left-hand side in close proximity to the last set of double bands in C2; C4 group 18 - the very fine band is missing drawn between the first and third bands of this group; D1 group 19 - the four bands of this group appear as tightly synapsed double bands and not as single bands as depicted by Keyl (op. cit.); D2 groups 22/23 - the major bands in these groups are strongly heterochromatic which makes it difficult to site the four fine bands between them, these can be more easily seen in the Malpighian tubule chromosome than in the salivary gland chromosome; D3 group 25 - the three bands of this group are more tightly synapsed than depicted by Keyl (op. cit.); E1 group 28 - three additional bands can be seen to the right of the second band drawn by

Keyl (op. cit.) for a total of five bands in this group.

In comparing the EM micrographs of the salivary gland and Malpighian tubule chromosomes in Figure 32 as well as the remaining sections of the two chromosomes, it was possible to find a band by band homology (or structure by structure in the case of tissue specific puffs) between the two tissues. However, the following physical differences could be noted in the preparations analyzed which may be related to the lower degree of polyteny in the Malpighian tubule chromosomes:

(1) The average width calculated for the salivary gland chromosome was 28.5 μm and 10.7 μm for the Malpighian tubule chromosome. The salivary gland chromosome depicted has, therefore, a width approximately 2.7 times that of the Malpighian chromosome. The total length of the chromosomes, from which the sections were taken, varied slightly at 406.2 μm for the salivary gland chromosome and 389.2 μm for the Malpighian tubule chromosome.

(2) Only the Malpighian tubule chromosome exhibits ectopic pairing. (For example, in Fig. 32, between the heterochromatic bands in groups 23 and 24 of division D2).

(3) Double bands are better separated in the Malpighian tubule chromosome, together with slight variations in the interband lengths.

(4) Only the the Malpighian tubule chromosome displays large tissue specific puffs. (For example, in Fig. 32, in division D3 group 26).

In comparing the overall banding patterns of the Malpighian tubule and the salivary gland chromosomes I, II, and III (Figures 23 - 25 and 27 - 29), the same band by band

homology (or structure by structure in the case of tissue specific puffs) was found as demonstrated in Figure 32. Due to the large Balbiani rings exhibited by the salivary gland chromosome IV, an exact, band by band, determination of the constancy of the banding pattern could not be carried out for that chromosome. In the majority of cases, the band/interband spacing between the chromosomes appeared to be approximately proportional between the chromosomes of the two tissues. In some cases, however, the spacing varied considerably to that of the respective salivary gland chromosome (e.g. division C6 in chromosome II, see Figures 24 and 28). This appears to be primarily the result of an elongation of the interbands in the Malpighian tubule chromosomes. Probably, because of the lower degree of polyteny, the Malpighian tubule chromosomes were, in general, slightly shorter than the salivary gland chromosomes (with the exception of chromosome III) and had widths approximately $1/2$ to $1/3$ those of the respective salivary gland chromosomes (for comparative results in LM analyses see Pavan and Breuer (1952) for Drosophila and Beermann (1952) for Chironomus).

In contrast to the salivary gland chromosomes, chromosome II of the Malpighian tubules was found to be longer than chromosome I. In the EM photo maps of the Malpighian tubule chromosomes (Figures 27 - 30), chromosome I has a length of 388 μm and an average width of ca. 12.5 μm , chromosome II has a length of 443 μm and an average width of ca. 10.7 μm , chromosome III has a length of 350 μm and average widths of ca. 10.7 μm for the right arm and 7 μm for the left arm, and chromosome IV has a length of 106 μm . (For the comparable salivary gland measurements see pp. 104 - 105)

(C) Physical appearance of intercalary heterochromatin (IH)

As noted section 5.4.1. ("Concerning intercalary heterochromatin), the physical appearance of intercalary hetero-

chromatin is characterized by "ectopic pairing", "weak points", and "constrictions". Whereas the salivary gland chromosomes of Ch. th. piger are relatively devoid of signs of intercalary DNA under normal culture conditions, the Malpighian tubule chromosomes display, much to the contrary, a cytology much more reminiscent of what one would expect from typical Drosophila salivary gland chromosomes. This includes extensive ectopic pairing, weak points, and constrictions along with a strong condensation of individual bands.

In the salivary glands of Chironomus tentans, Ch. thummi thummi, and Ch. th. piger, an occasional unspecific terminal adhesion of the chromosomes may be seen (Bauer, 1935; Beermann, 1952; Whitmore, unpublished data), but this is an extremely infrequent occurrence. Chromosomal breaks may be induced in the salivary glands by subjecting them to X-ray treatment (Keyl, 1958), through the use of malachite green (Keyl and Werth, 1959), by the incubation with 5-fluoruracil-2-deoxyribosid (FUDR) (Hägele, 1971) or in Ch. th. th. (male) x Ch. th. piger (female) hybrids (Hägele, 1984). Hägele (1971) was able to demonstrate that the breaks in Ch. thummi chromosomes occurred mainly in sections containing late replicating bands of high DNA content and presented histograms showing the distribution of the chromosomal breaks in relationship to ³H-thymidine autoradiographical labeling patterns. The chromosomal weak points which I determined by the Malpighian tubule chromosomes are in accordance with induced break points found by Hägele (op. cit.) for the salivary glands of Chironomus thummi.

It was noted in the electron microscopic photo maps of the salivary gland chromosomes in Drosophila hydei (Results 5.2.) that one could differentiate between two types of constrictions. Type-1 constrictions were found exclusively at distinct bands or band groups whereas the threadlike type-2 constrictions were found in interbands or sections with faint chromosome bands. The cytological appearance of

these differences has been detected only by the SSP chromosome preparation technique in which the chromosomes are not stretched by a squashing process and in which all of the DNA material of a chromosomal section is depicted in the individual preparation. In comparing the electron micrographs of the Malpighian tubule and salivary gland chromosomes of Ch. th. piger (Figures 23 - 26 and Figures 27 - 30), both type-1 and type-2 constrictions could be seen in the Malpighian tubule chromosomes. In the salivary gland chromosomes, however, only type-1 constrictions were observed.

Tables 9 - 12 summarize the characteristic appearance of intercalary heterochromatin in the form of ectopic pairing, weak points, and constrictions for the individual divisions of the Malpighian tubule and salivary gland chromosomes of Chironomus thummi piger.

(D) Telomeres

In contrast to the characteristically flaired telomeres seen in both SSP and squash preparations of the salivary gland chromosomes of Ch. th. piger, the Malpighian tubule SSP chromosomes preparations exhibit compact and well-defined telomeric regions. The typical structure and forms of the telomeres of chromosomes I, II, and III of the Malpighian tubules are depicted in Figures 33 - 35. The telomere of the right arm of chromosome III showed routinely ectopic pairing with one or both of the first two major groups of bands in division A1 (see Figures 29 and 34A).

Seen for the first time, were eight additional bands in chromosome I noted directly following the telomere in division G3 (see Figure 33). These six small to medium size bands and two minibands are not depicted in the chromosome maps of the salivary gland chromosomes of Chironomus th. thummi (Bauer, 1935; Keyl, 1957; Hägele, 1970) and also could not be found, as of yet, in electron micrographs of

SSP preparations of Ch. th. piger salivary glands. At this point, it is yet not clear whether it is the flaired nature of the salivary gland chromosome which hinders the visibility of these bands or if this is a case of band modulations or if there possibly exists a tissue specific terminal deficiency in the salivary gland chromosome.

It should be noted here that it is still not assertent whether or not terminal deficiencies actually exist and that there is still relatively little known concerning the nature of the polytene telomeres. Roberts (1979) studied in Drosophila melanogaster a number of stocks which have been described as containing such terminal deficiencies. He proposed that the boundry of polytenized and nonpolytenized DNA sequences may vary in the telomeric region. Thus, stocks exhibiting terminal deficiencies possess underreplicated terminal bands which are cytologically difficult to locate. Roberts (1988) has also noted band modulations in comparing the tip of the midgut chromosome arm 5S with that of the salivary gland chromosome in Drosophila gibberosa. Here, he interprets band modulation as "visible manifestations of the differential chromatin compaction of bands and adjacent interbands associated with differential gene expression."

It would be of future interest, therefore, to microclone these bands of the Malpighian tubule chromosome in Chironomus th. piger and to examine the extent of their presence in the salivary glands through hybridization studies.

The significance of the results presented in section 5.4 may be briefly summarized as following:

The EM investigation in this section verifies the generality of the LM observations of Beermann (1952) on Chironomus tentans and the model of dynamic organization of polytene chromosomes proposed by Zhimulev et al. (1981) in as so far as the idea of

band splitting is concerned. For the first time, it has been conclusively demonstrated at the EM level that band by band (or structure by structure in the case of tissue specific puffs) homology can exist between chromosomes of different tissues. But, that there also can occur a modulation of the bands "band splitting" of the identical structure in both tissues and that the modulation is not limited to just one or the other tissue.

Furthermore, the EM observations indicate, for the first time, that the physical manifestation of transcriptionally active regions in the form of visible puffs may not only be dependent on the level of transcription, but also on the degree of polyteny in the tissue studied.



Figure 21. Demonstrative comparison of a constriction in *Ch. th. piger* salivary gland chromosome II, division 2A, between (A) a squash preparation, 1,600x and (B) an EM micrograph of a SSP chromosome preparation, 3,200x. The SSP chromosome shows a ca. 3.8 fold lateral degree of spreading in comparison to the squash preparation of comparable polyploidy. Reference numbers according to Hägele (1970). (Source: Whitmore, unpublished)



Figure 22. Demonstrative comparison of Ch. th. piger salivary gland chromosome II, divisions 4Bg - 5Be, between (A) a squash preparation, 1,600x and (B) an EM micrograph of a well spread SSP chromosome preparation, 3,200x. The SSP chromosome shows a ca. 5.3 fold lateral degree of spreading in comparison to the squash preparation of comparable polyploidy. Reference numbers according to Hägele (1970). (Source: Whitmore, unpublished)



Figure 23. Electron microscopic map of salivary gland chromosome I of Chironomus thummi piger. 1,200x. (Source: Whitmore, unpublished)

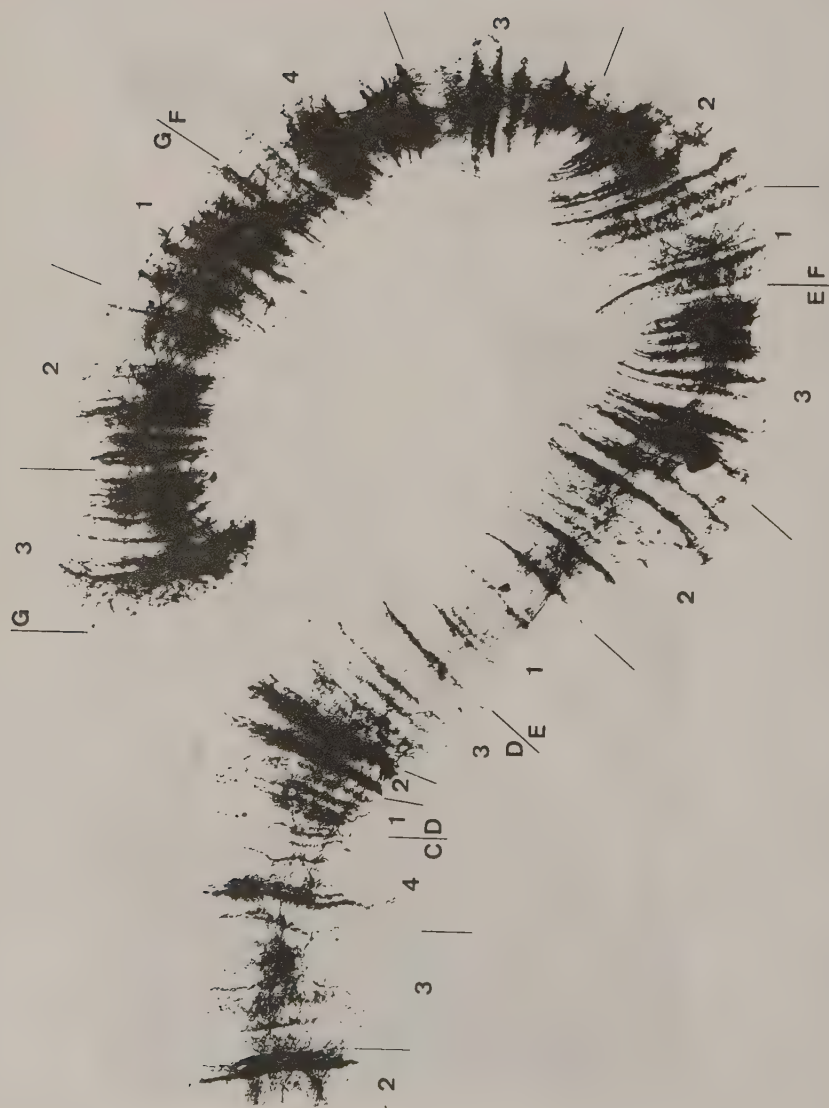


Figure 23 (continued)

CHROMOSOME II



Figure 24. Electron microscopic map of salivary gland chromosome II of *Chironomus thummi piger*. 1,200x. (Source: Whitmore, unpublished)



Figure 24 (continued)

CHROMOSOME III

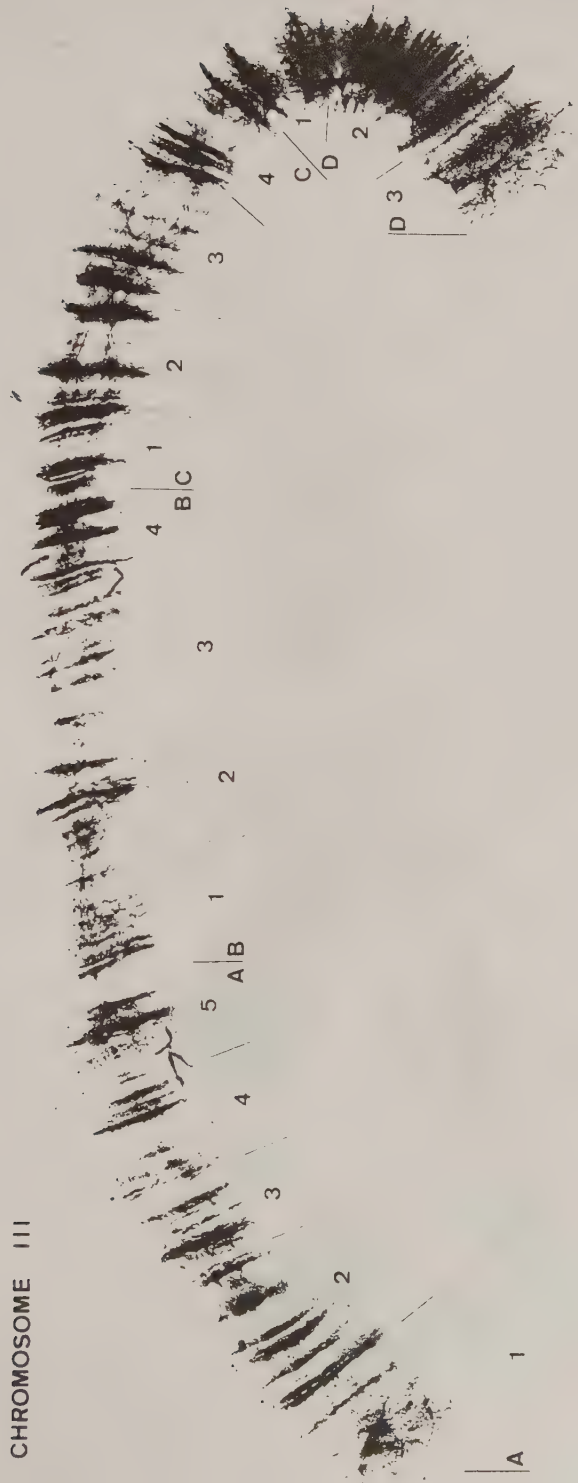


Figure 25. Electron microscopic map of salivary gland chromosome III of Chironomus thummi piger. 1,200x. (Source: Whitmore, unpublished)

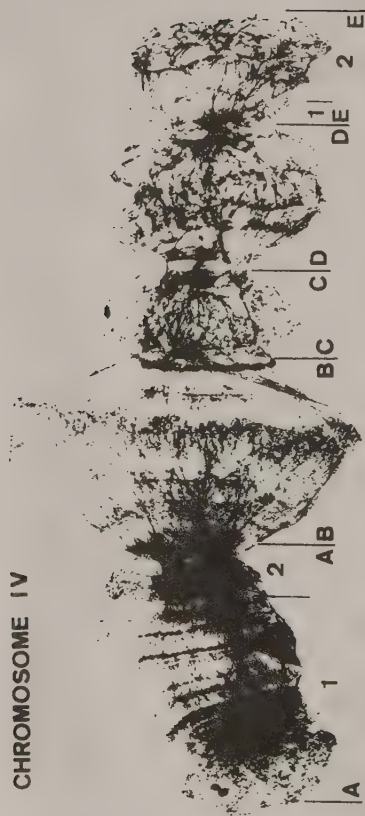


Figure 26. Electron microscopic map of salivary gland chromosome IV of Chironomus thummi piger. 1,200x. (Source: Whitmore, unpublished)

CHROMOSOME I



Figure 27. Electron microscopic map of Malpighian tubule chromosome I of *Chironomus thummi piger*. 2,800x. (Source: Whitmore, unpublished)



Figure 27 (continued)



Figure 27 (continued)



Figure 27 (continued)

CHROMOSOME II

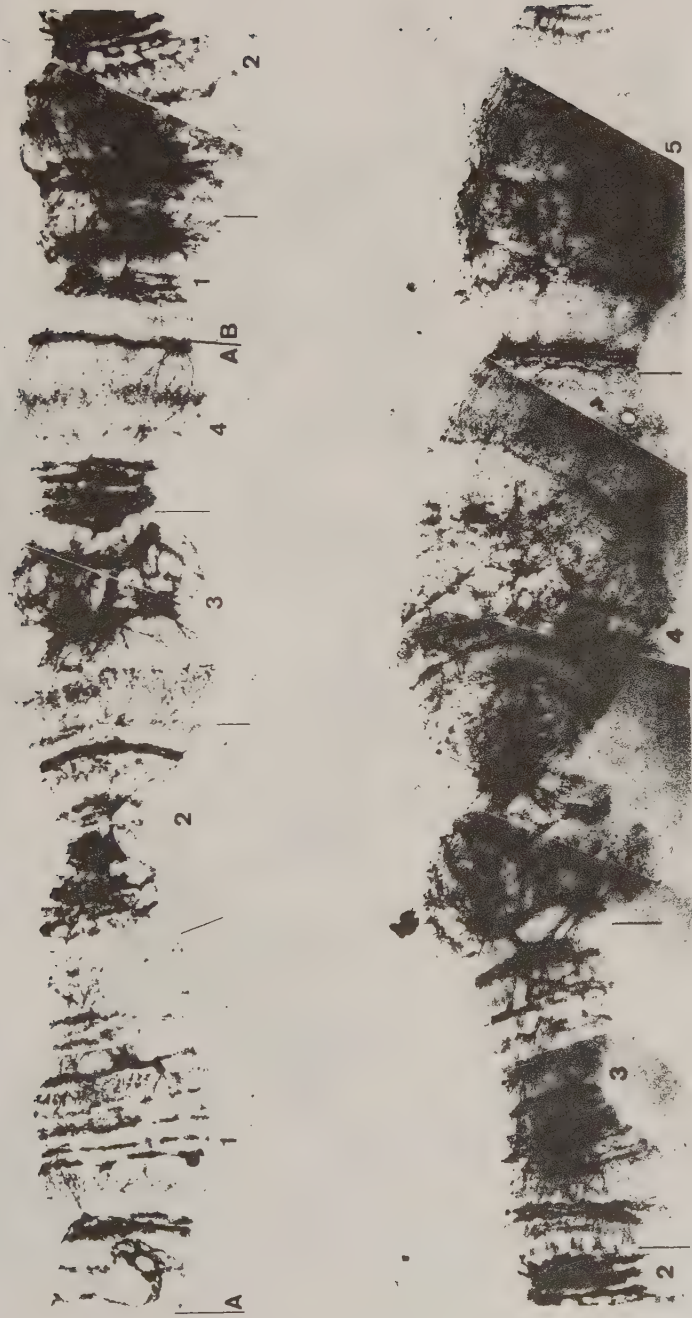


Figure 28. Electron microscopic map of Malpighian tubule chromosome II of Chironomus thummi piger. 2,800x. (Source: Whitmore, unpublished)

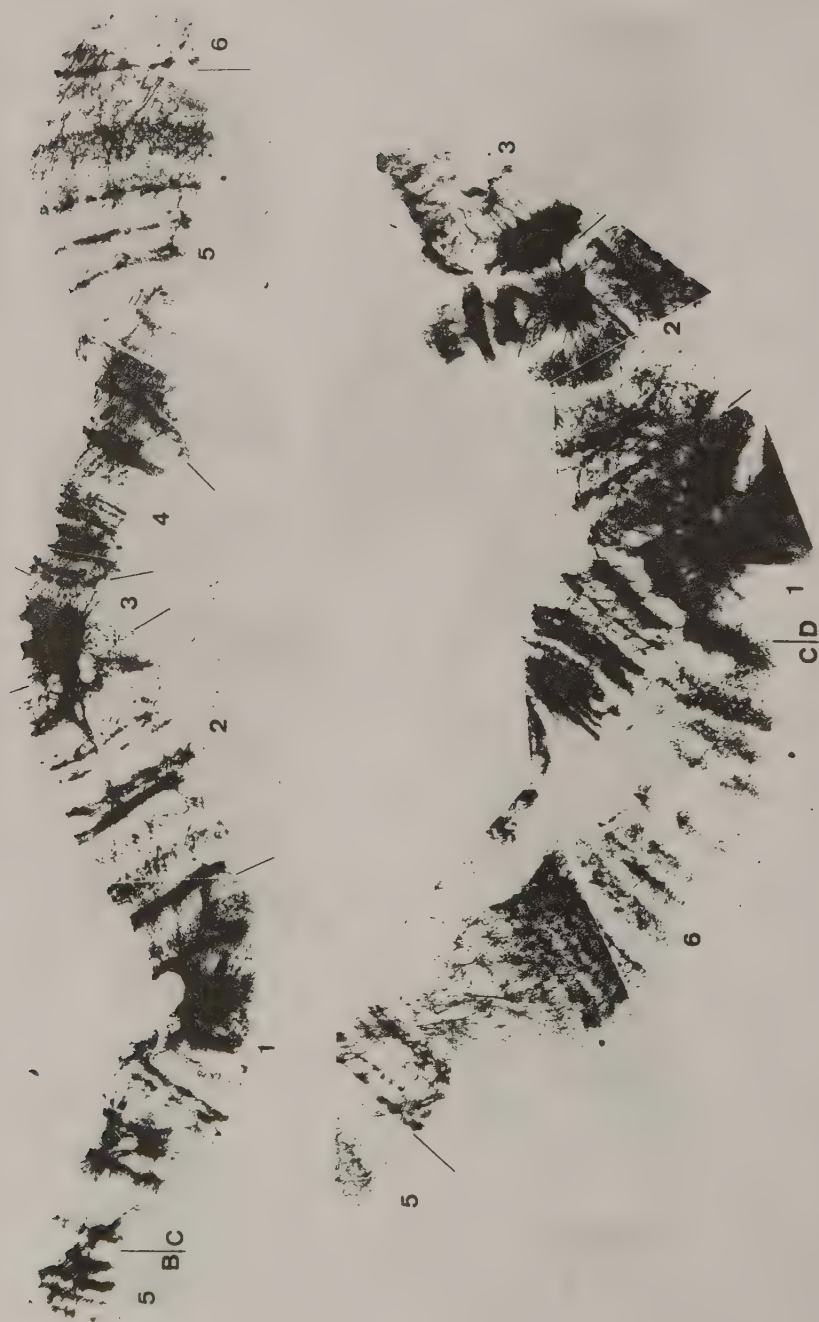


Figure 28 (continued)



Figure 28 (continued)



Figure 29. Electron microscopic map of Malpighian tubule chromosome III of *Chironomus thummi piger*. 2,800x. (Source: Whitmore, unpublished)



Figure 29 (continued)



Figure 29 (continued)

CHROMOSOME IV



Figure 30. Electron microscopic map of Malpighian tubule chromosome IV of Chironomus thummi piger.
2,800x. (Source: Whitmore, unpublished)

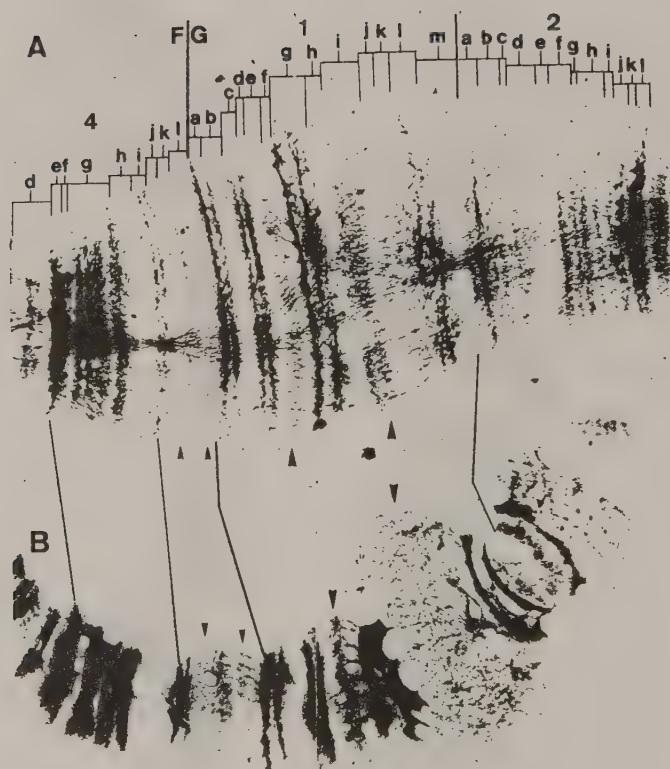


Figure 31. Comparative electron micrographs of chromosome I, divisions F4d - G21, in Chironomus thummi piger from (A) the salivary glands, 1,340x and (B) the Malpighian tubules, 2,800x. Large arrows indicate common puffed regions in both tissues at G1e and G1i. Small arrows indicate regions which contain modulating band structures (see text for details). Reference numbers according to Hägele (1970). (Source: Whitmore, unpublished)

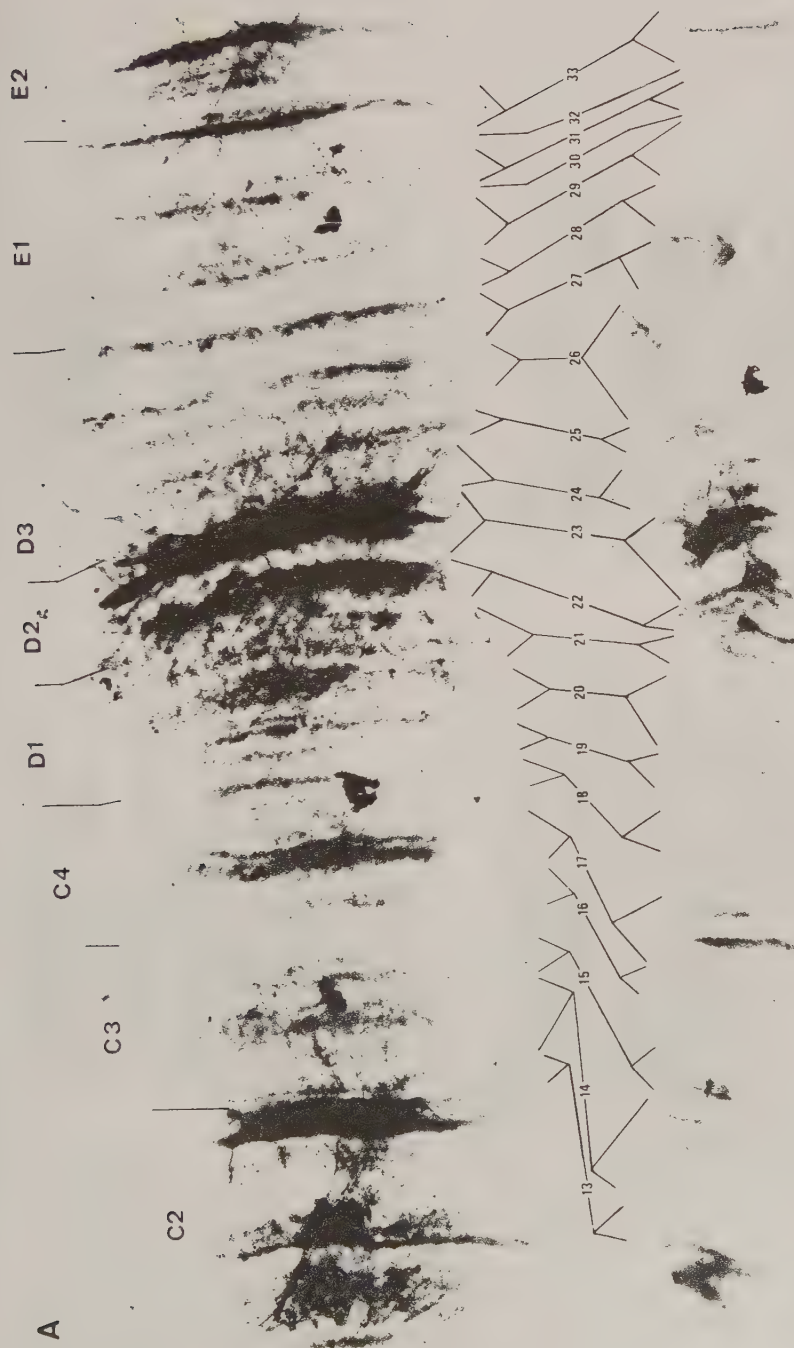


Figure 32. Comparative electron micrographs of chromosome I, divisions C3-E1, in *Chironomus thummi* pliger from (A) the salivary glands and (B) the Malpighian tubules. Both preparations are from the same larva and depicted at the same magnification. 2,800x. Reference numbers according to Keyl (1957). (Source: Whitmore, unpublished)

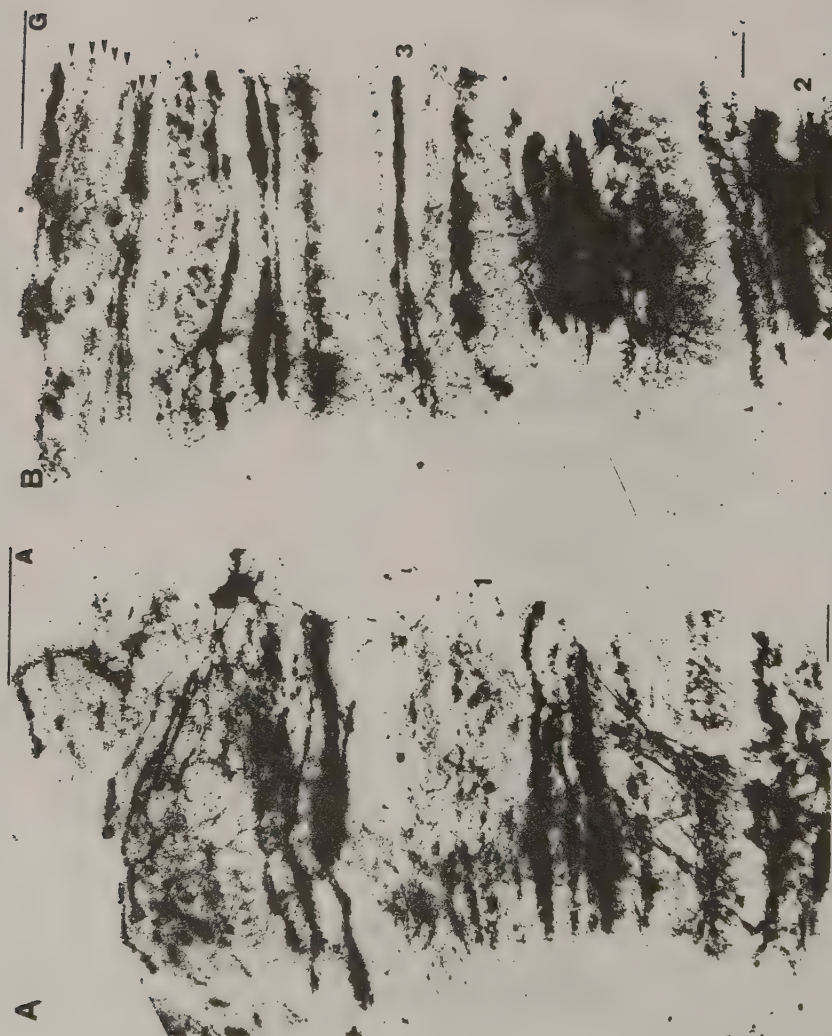


Figure 33. Electron micrographs of the telomeric sections of *Chironomus thummi piger* Malpighian tubule chromosome I. (A) Arm A. (B) Arm B. Arrows indicate additional bands not depicted in the chromosome maps of *Ch. th.* (Keyl, 1957; Hägele, 1970). 5,600X. (Source: Whitmore, unpublished)

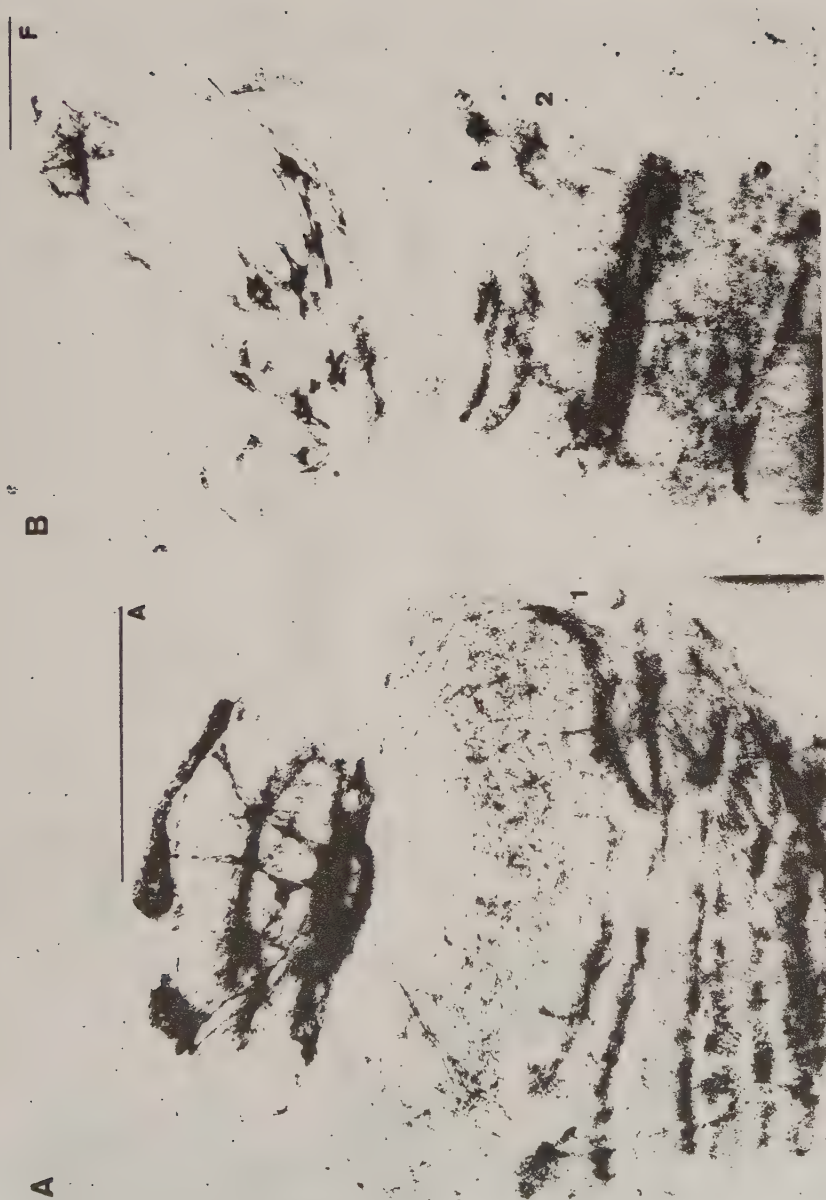


Figure 34. Electron micrographs of the telomeric sections of *Chironomus thummi piger* Malpighian tubule chromosome II. (A) Arm C. (B) Arm D. 5,600x. (Source: Whitmore, unpublished)

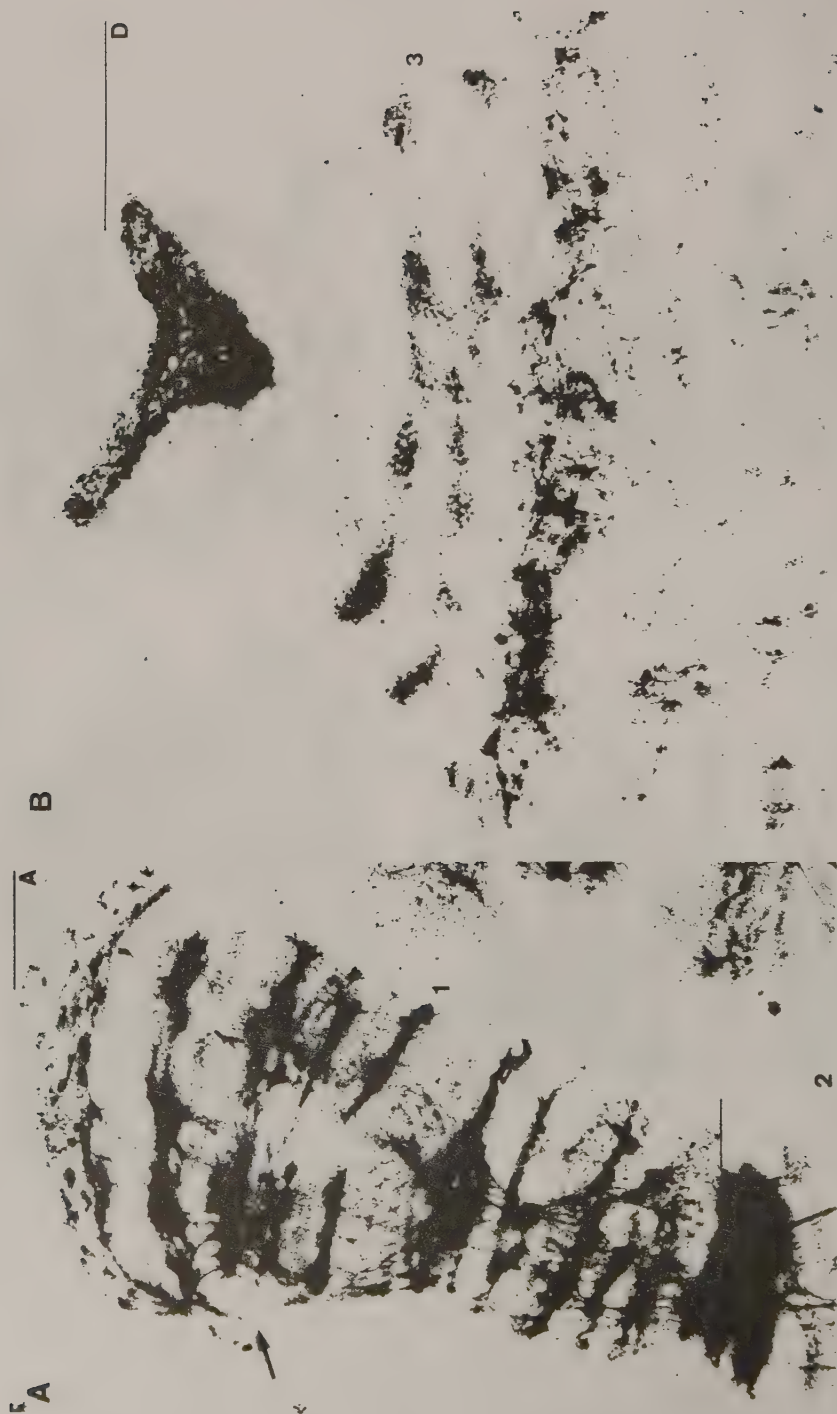


Figure 35. Electron micrographs of the telomeric sections of *Chironomus thummi piger* Malpighian tubule chromosome III. (A) Arm E. Arrow indicates intercalary chromatin threads "ectopic pairing". (B) Arm F. 5,600x. (Source: Whitmore, unpublished)

Table 5. Prominent puffs in Malpighian tubule chromosome I of Chironomus th. piger in fourth instar larvae

Chromosome division	Tissue specific puffs ¹	Common puffs ²
A1	(3)	(1)
A2	1	1
A3	-	1
A4	2	(1)
B1	-	-
B2	-	1, (2)
B3	-	1
B4	-	(3)
C1	-	(1)
C2	1	-
C3	-	3
C4	-	-
D1	-	-
D2	-	-
D3	1	-
E1	-	2
E2	-	-
E3	-	-
F1	(1)	-
F2	-	2
F3	-	(1)
F4	1	-
G1	-	2*
G2	-	3
G3	2	(1)
Totals	12	26

¹Puffs which occur solely in the Malpighian tubule chromosomes at this stage of larval development.

²Prominent Malpighian tubule chromosome puffs which are common to transcriptionally active sites in the salivary gland chromosomes as indicated by band decondensation in SSP chromosome preparations of Ch. th. piger and/or through ³H-uridine incorporation in Ch. th. th. (Kiknadze, 1981).

*Prominent salivary gland puff(s). Parentheses indicate less prominent puffs. Data based on a minimum of 2 - 3 electron microscopic SSP Malpighian tubule and salivary gland chromosome preparations.

Table 6. Prominent puffs in Malpighian tubule chromosome II of Chironomus th. piger in fourth instar larvae

Chromosome division	Tissue specific puffs ¹	Common puffs ²
A1	1	1
A2	-	-
A3	-	2+, (1)
A4	1	-
B1	-	1
B2	-	1
B3	-	-
B4	-	3+
B5	1	2
C1	(3)	-
C2	-	-
C3	-	-
C4	-	-
C5	-	1
C6	(1)	-
D1	-	1
D2	-	-
D3	1	-
D4	-	1
E1	-	1
E2	1	-
E3	-	1
E4	-	1
F1	1	-
F2	1	(4)
Totals	11	21

See legend of Table 5.

Table 7. Prominent puffs in Malpighian tubule chromosome III of Chironomus th. piger in fourth instar larvae

Chromosome division Tissue specific puffs¹ Common puffs²

A1	1	1
A2	-	2 ⁺ , (1)
A3	1	1
A4	-	2
A5	-	3
B1	-	(2)
B2	-	-
B3	1	1 ⁺
B4	1	-
C1	-	1
C2	-	1
C3	-	2
C4	-	(1)
D1	-	2
D2	1	2
D3	2	-
Totals	7	22

See legend of Table 5.

Table 8. Prominent puffs in Malpighian tubule chromosome IV of Chironomus th. piger in fourth instar larvae

Chromosome division	Tissue specific puffs ¹	Common puffs ²
A1	3	-
A2	1	-
B	-	1+
C	-	1+
D	-	(nucleous)
E1	-	-
E2	-	-
Totals	4	2

See legend of Table 5. Note: The common Malpighian tubule and salivary gland chromosome puffs in divisions B and C of chromosome IV are of the Balbiani-ring type (for further details see text).

Table 9. Characteristic appearance of intercalary heterochromatin in Malpighian tubule and salivary gland chromosome I of Chironomus thummi piger

Chromosome division	Ectopic pairing		Weak points		Constrictions	
	M.T.	S.G.	M.T.	S.G.	M.T.	S.G.
A1	-	-	-	-	-	-
A2	(+)	-	(+)	-	-	-
A3	-	-	-	-	+	-
A4	-	-	-	-	+	+
B1	+	-	+	-	+	+
B2	-	-	+	-	-	-
B3	+	-	-	-	+	-
B4	-	-	-	-	-	-
C1	-	-	-	-	-	-
C2	+	-	(+)	-	+	-
C3	-	-	-	-	-	-
C4	-	-	-	-	-	-
D1	+	-	-	-	+	-
D2	+	-	-	-	+	-
D3	-	-	-	-	-	-
E1	-	-	-	-	-	-
E2	+	-	-	-	+	-
E3	+	-	+	-	+	(+)
F1	-	-	-	-	-	-
F2	(+)	-	+	-	+	(+)
F3	(+)	-	-	-	+	(+)
F4	+	-	-	-	(+)	(+)
G1	(+)	-	-	(+)	+	-
G2	(+)	-	-	-	+	(+)
G3	-	-	-	-	+	(+)

M.T.- Malpighian tubule chromosome.

S.G.- Salivary gland chromosome.

Parentheses indicate that the appearance of the intercalary heterochromatin is less prominent.

Data based on a minimum of 2 - 3 electron microscopic SSP Malpighian tubule and salivary gland chromosome preparations.

Note: In a few rare cases, telomeric ectopic pairing was seen in both the salivary gland and Malpighian tubule chromosomes. Here, the salivary gland chromosomes showed ectopic pairing between two different chromosomes, whereas the Malpighian tubule chromosomes displayed an end to end ectopic pairing of the same chromosome.

Table 10. Characteristic appearance of intercalary heterochromatin in Malpighian tubule and salivary gland chromosome II of Chironomus thummi piger

Chromosome division	Ectopic pairing		Weak points		Constrictions	
	M.T.	S.G.	M.T.	S.G.	M.T.	S.G.
A1	-	-	-	-	(+)	-
A2	(+)	-	-	-	+	+
A3	(+)	-	-	-	(+)	-
A4	-	-	-	-	(+)	-
B1	-	-	-	-	(+)	-
B2	-	-	-	-	(+)	-
B3	(+)	-	-	-	+	-
B4	(+)	-	(+)	-	+	-
B5	+	-	(+)	-	+	-
C1	+	-	(+)	-	(+)	-
C2	+	-	-	-	-	-
C3	+	-	(+)	-	+	-
C4	-	-	-	-	+	-
C5	-	-	-	-	-	-
C6	-	-	-	-	(+)	(+)
D1	+	-	(+)	-	+	-
D2	+	-	(+)	-	+	-
D3	+	-	(+)	-	+	-
D4	+	-	-	-	(+)	(+)
E1	+	-	+	-	(+)	(+)
E2	-	-	-	-	(+)	(+)
E3	-	-	-	-	-	-
E4	-	-	-	-	+	(+)
F1	-	-	-	-	-	(+)
F2	-	-	-	-	+	(+)

See legend of Table 9.

Table 11. Characteristic appearance of intercalary heterochromatin in Malpighian tubule and salivary gland chromosome III of Chironomus thummi piger

Chromosome division	Ectopic pairing		Weak points		Constrictions	
	M.T.	S.G.	M.T.	S.G.	M.T.	S.G.
A1	+	-	-	-	-	-
A2	-	-	-	-	-	-
A3	-	-	+	-	+	-
A4	-	-	+	-	+	-
A5	-	-	-	-	-	-
B1	-	-	-	-	-	-
B2	+	-	-	-	-	-
B3	+	-	-	-	+	-
B4	(+)	-	-	-	+	(+)
C1	-	-	-	-	+	-
C2	-	-	-	-	-	-
C3	-	-	-	-	(+)	(+)
C4	+	-	-	-	-	-
D1	+	-	-	-	(+)	-
D2	+	-	-	-	+	(+)
D3	-	-	-	-	-	-

See legend of Table 9.

Table 12. Characteristic appearance of intercalary heterochromatin in Malpighian tubule and salivary gland chromosome IV of Chironomus thummi piger

Chromosome division	Ectopic pairing		Weak points		Constrictions	
	M.T.	S.G.	M.T.	S.G.	M.T.	S.G.
A1	(+)	-	-	-	+	(+)
A2	(+)	-	+	-	+	+
B	(+)	-	-	-	-	-
C	(+)	-	-	-	+	(+)
D	(+)	-	-	-	+	(+)
E1	(+)	-	(+)	-	+	(+)
E2	(+)	-	-	-	+	-

See legend of Table 9.

5.5. A novel whole mount transfer method of in situ hybridized and immunogold labeled surface-spread polytene chromosomes onto grids for electron microscopic (EM) study

(Sources: Based on Whitmore, unpublished and XI)

As detailed in Methods (sections 4.3.3., 4.3.4., and 4.4.), DNA-probes can be labeled through the use of biotin-labeled nucleotides. After in situ hybridization of the biotinylated probes to chromosomes, the biotin molecules serve as antigens binding primary antibodies (e.g. rabbit anti-biotin antibodies). The binding sites of the primary antibody can be visualized through the use of a secondary antibody (e.g. goat anti-rabbit IgG) which is conjugated to, for example, fluorescein isothiocyanate (FITC) for light microscopy (LM) or colloidal gold for electron microscopy (EM). A particular advantage of colloidal gold is the relatively small size in comparison to the silver grain size of autoradiographic studies where DNA-probes are labeled with radioisotopes. The 20 and 40 nm immunogold reagents used here are about 1/10 - 1/20 the size of an average fine silver grain.

There are, at the present, two whole mount techniques for the transferring of in situ hybridized and gold labeled polytene chromosomes onto grids for electron microscopy. The first one was introduced by Wu and Davidson (1981), and the second is the one which I have now developed especially for SSP chromosomes and described in Methods (section 4.3.5.).

The transfer technique of Wu and Davidson (op. cit.) entails, in brief: 1) the extensive washing of the chromosomes (prepared on chrome alum subbed slides) in a buffer solution, rinsing in H_2O , and air drying, 2) coating with

Parlodion and air drying, 3) cutting out of the area around the chromosome(s) with a razor blade and placing an EM grid over the chromosome(s), 4) floating off the Parlodion film, chromosome(s), and grid on the surface of hydrofluoric acid, 5) picking up the grid on a Saran Wrap covered beaker glass, and carefully rinsing with H_2O .

Kress et al. (1985), working in conjunction with and in Davidson's laboratory, used successfully a modified version of the Wu and Davidson technique (op. cit.) for the transfer of gold labeled SSP chromosomes of Drosophila melanogaster. I had also originally intended to use their technique, but I continually ran into several major technical problems which have also hindered other research groups from using this method (see Saura, 1986). These problems are basically that, usually, the film does not detach from the slide and when it does, it remains stuck around the area where the chromosomes are located or when the film does completely float off, the chromosome(s) remain affixed to the microscope slide. More than several months of fruitless effort was put into trying to get this technique to work on any sort of basis, not even to mention the thought of a routine one. After personal communications with Kress, it was apparent that another transfer method was needed if work in this field was intended to be carried out on a routine basis.

In this section, the novel technique introduced in Methods section 4.3.5. is used for transferring in situ hybridized and immunogold labeled SSP chromosomes onto grids for electron microscopy. Located are a repetitive DNA sequence (the "Cla-element") and a "housekeeping" gene (the haemoglobin IV gene) in Chironomus thummi. Comparisons are made to in situ hybridized and FITC (fluorescein isothiocyanate)

labeled squash preparations and a ^3H -labeled autoradiographic light microscopic SSP chromosome preparation.

5.5.1. "Cla-element"

For demonstration purposes, the so-called "Cla-element" was chosen to work with (Schmidt, 1981; 1984). Its name is derived from the restriction site it has for the endonuclease Cla I and belongs to a family of repetitive elements found in Chironomus. This repetitive element has been intensively studied both cytologically, at the light microscopic level, and molecular genetically (see Schmidt op. cit. and references therein). It should provide, therefore, a good basis to work with. The DNA-probe used here was a trimere of the 120-base repetitive DNA sequence isolated from the genome of Chironomus thummi.

Immunofluorescence light microscopy of a salivary gland squash preparation

Figure 36 depicts the results of a typical in situ hybridization with a squash preparation of Ch. th. thummi where the hybridization sites of the biotinized "Cla"-probe were detected fluorimetrically using rabbit anti-biotin antibodies and affinity isolated FITC-labeled goat anti-rabbit IgG as described in Methods section 4.4. Numerous positive responses can be seen on each of the salivary gland chromosomes I and III. For a correlation between the position of the primary fluorescence and its chromosomal location it is usually necessary to Giemsa stain such preparations afterwards, photograph the chromosomes again, and then compare the photo taken with fluorescence microscopy with that of the light microscopically taken one.

Light microscopic autoradiography of a SSP salivary gland chromosome I preparation

Figure 37 depicts the results of an in situ hybridization with a SSP chromosome preparation of Ch. th. piger salivary gland chromosome I. Here, the sites of the ^3H -labeled "Cla"-probe were detected autoradiographically using Kodak AR-10 stripping film. A 4,160x enlargement of this preparation was used to calculate the average silver grain size and density of the labeling. The average silver grain size was determined as $0.38 \pm 0.04 \mu\text{m}$ ($n = 25$). The average labeling density over the centromeric region was 2.06 ± 1.01 silver grains / μm^2 ($n = 157$), whereby areas showing no labeling were not included in the calculation. From the 157 labeled areas, the highest number of silver grains found in any μm^2 area was 6.

Electron microscopic immunogold labeling of SSP salivary gland chromosome I preparations

Figure 38A is a phase contrast light microscopic photo of a SSP chromosome preparation of chromosome I from Chironomus th. thummi before being denatured and used for hybridization. The chromosome depicted has a ca. 2.8 fold longitudinal and 3.5 fold lateral degree of spreading in comparison to squash preparations of comparable polyteny.

Figure 38B is an electron micrograph of the area in the centromeric region indicated by brackets in Figure 38A from the same chromosome after hybridization with the biotinized "Cla"-probe and labeling of the hybridization sites with rabbit anti-biotin and immunogold reagent (GAR G20). Determined, from 20,000x enlargements, were the average size of the IgG gold conjugate, the average labeling in the centromeric region and the significance of the labeling. The average size of the IgG gold spheres was $0.0246 \pm 0.0043 \mu\text{m}$ (ca. 25 nm, $n = 25$). The average labeling density was calculated as being 5.4 ± 3 IgG gold spheres / μm^2 ($n = 27$).

Increasing the number of areas used in the calculation did not appreciably increase or decrease the average labeling density (for example, by $n = 136$ an average labeling density was found of 5.1 ± 2.6 IgG gold spheres / μm^2). The highest number of IgG gold spheres found in any μm^2 area was 13. To determine the level of significance of the labeling, the average amount of labeling in the next two interbands closest to the centromeric area [D1h and D1j in Hägele's map of Ch. th. thummi (1970)] was used and a level of significance of $p < 0.001$ calculated. This means that the labeling found in the centromeric area is very significant.

In order to determine the state of the chromatin condensation (i.e. DNA packing ratio) at the sites of the labeling, 200,000x enlargements were used. A cutout from Figure 38C (from chromosome subdivision D2c) is depicted by such an enlargement in Figure 38D. Arrows indicate: (a) Typical immunogold particles with ca. 25 nm diameter. (b) A thin DNP fiber of ca. 3.5 nm thickness with a beaded structure. Up to about five such fibers with thicknesses of ca. 3 - 5 nm can be seen attached to each of the immunogold particles. (c) Ca. $6 \times 10 - 11 \times 14$ nm particles with very thin fibers (ca. 2 - 2.5 nm) attached. As indicated from their size, these represent most likely nucleosome structures to which DNA fibers are attached exhibiting a minimal amount of associated protein. (d) Very thin fibers of ca. 2 - 2.5 nm thickness. (e) A ca. 12.5 - 15 nm diameter ring or elliptical shaped structure which is apparently composed of four ca. 6 - 7 nm elements. From the size, this appears to be one subunit of a 12.5 - 15 nm unit fiber. (f) Ca. 5 - 6 nm thick DNP fibers running parallel to each other. These fibers each seem to be composed of two finer 2 - 2.5 nm fibers.

Although, the SSP chromosome preparation itself appears to be structurally in a transitional state, it can be concluded from the measurements of the DNP fibers in Figure

38D, that at the sites of labeling, each chromatin fiber attached to an IgG gold particle represents, a totally extended single DNA double helix with associated proteins.

In Figure 39A an electron micrograph is shown from division A3 of Chironomus th. thummi chromosome I where the chromosome was hybridized with the same "Cla"-probe as above, but here the hybridized sites were labeled with 40 nm immunogold reagent. The two bands in the subdivisions A3b and one of the bands in division A3d at position A3d2 show labeling. The areas between the labeled sites are relatively free of gold marker. Figure 39B is a cutout of subdivisions A3a-b at a higher magnification. A number of gold conglomerates can be seen, i.e. groups of ca. 4 - 12 gold particles in close association. This is often a problem when using immunogold reagents with a particle size of 40 nm or larger. An additional centrifugation step was found to be helpful in reducing the number of such conglomerates as described in Methods section 4.3.4. ("Detection of hybridized probe"). The average labeling in the bands of subdivisions A3b was 7 ± 4.1 IgG gold spheres / μm^2 ($n = 22$). The highest number of spheres found in any μm^2 area was 17. The level of significance of the labeling was found to be very significant ($p < 0.001$) in comparison to the control area [the interband denoted in A3a of Hägele's map of Ch. th. thummi (1970)].

In comparing the LM and EM results it is apparent that even though the labeling is ca. 60 - 70% higher in EM than in LM preparations, it still appears, at a first glance, heavier in the LM squash preparations because of the larger size of the silver grains. The definite advantage of the EM gold labeling is in the clear delineation between what is labeled (in this case the dark banded material), and what is not (the interband material). This type of resolution is not possible even in the most highly magnified LM preparations.

By the immunofluorescence LM preparations, one must take into consideration that here the total emittance of the fluochrome particle is being photographically recorded and not the point of emittance itself. In other words, just as a common light bulb fills, for example, a room with light, what one is observing is the well lit room and not the source of the light, namely the light bulb. Due to this amplified picture, the immunofluorescence procedure represents a very sensitive and quick LM procedure for the preliminary mapping of genes on polytene chromosomes (see Langer-Safer et al., 1982). It still lacks, however, the resolution necessary for an exact structural localization as can be seen in the EM gold labeling.

5.5.2. Haemoglobin IV gene

Antoine and Niessing (1984) isolated, cloned, and sequenced four closely linked intron-less haemoglobin genes (labeled as "A", "B", "C", and "D") from a genomic library of the midge Chironomus thummi thummi. Two of genes were coded for haemoglobin III and the others for haemoglobin IV. In both cases, the respective genes were transcribed from opposite DNA strands. Their results indicated further that this particular sequence of the genes was most likely to be present only once per haploid genome and that the four globin genes represented globin genes expressed in vivo. The genes are tandemly located on a 15.5 kilobase (kb) Ch. th. thummi insert DNA.

The 0.945-Kb fragment "D" coding for the haemoglobin IV gene was localized imunologically on squash preparations using a hybridized biotinized DNA probe, rabbit anti-biotin antibodies, and FITC-labeled affinity-purified goat anti-rabbit IgG (Whitmore, 1986). This was the first case of a low copy "housekeeping" or permanently active gene being located on polytene chromosomes by in situ hybridization.

The fragment "D" was chosen for a number of reasons for the hybridization experiments. Niessing (1985) reported that in addition to the sequence of globin genes described (Antoine and Niessing, 1984) a small number of other clones of two types were found positive for the haemoglobin genes studied. Their sequences differed from the one primarily investigated through minor substitution of one amino acid or otherwise. The possibility that each of the complete sequences containing these globin genes is present only once per haploid genome and perhaps on different positions on the same or different chromosomes, indicates that the theoretical possibility of hybridizing and receiving a strong positive response when using one of the entire segments containing all four genes is not overly favourable under most hybridization conditions. Using the 0.945-Kb fragment coding for the globin IV gene by itself, however, one could theoretically expect a hybridization to all genes coding for haemoglobin IV regardless of the slight differences between the entire globin DNA clones mentioned above, and also to all of the haemoglobin III genes with which the globin IV gene shows a homology of 83% (Pfletschinger et al., 1980).

Immunofluorescence light microscopy of salivary gland squash preparations

The LM hybridization results (Whitmore, op cit.) showed one positive response on each of the three major salivary gland chromosomes I, II, and III of Chironomus th. thummi. In each case the location appeared to be that of a band-inter-band area near the chromosome ends (telomeres): by chromosome I on arm B at position G3o, by chromosome II on arm C in region Alc, and by chromosome III on arm E at position A1b. The strongest and primary signal was observed on chromosome III. The signals on chromosomes I and II were quite weak in comparison, with that on chromosome I being the faintest. The primary location of the haemoglobin IV gene at the end of chromosome III, arm E, has been reconfirmed

by later authors for a number of chironomid species (Schmidt et al., 1988; Hankeln et al., 1988).

Figure 40A depicts the hybridization site of the biotin-labeled haemoglobin IV gene to the salivary gland chromosome III of a Ch. th. thummi squash preparation as detected by indirect immunofluorescence. For a correlation between the position of the primary fluorescence and its chromosomal location, each exposure taken with fluorescence microscopy was followed directly by a phase contrast exposure under otherwise identical conditions. After Giemsa staining of the preparations, the position as indicated by the phase contrast exposure was then determined using the chromosome map of Chironomus th. thummi (Hägele, 1970). Figure 40B is the identical chromosome after Giemsa staining indicating the same region.

The resolution of the methods was not adequate enough to permit locating on squash preparations the hybridized DNA to solely the band or interband region.

Electron microscopic immunogold labeling of SSP salivary gland chromosome III

Figure 41A shows a LM photo of a SSP chromosome preparation of chromosome III of Chironomus th. thummi before being used for in situ hybridization. The chromosome depicted has a ca. 2.7 fold longitudinal and 4.7 fold lateral degree of spreading in comparison to squash preparations of comparable polyteny.

Figure 41B is an electron micrograph of the area indicated by brackets in the LM photo after hybridization with biotin-labeled haemoglobin IV gene probe and subsequential double labeling of the hybridization site with 20 and 40 nm immunogold reagents. Labeling is seen over the two bands and the interband between them in subdivision A1b. The maximum length of the area covered by the labeling was ca. 4.5

- 5 μm . This is equal to ca. 2.9 - 3.2 kb DNA / μm (assuming that the DNA probe is hybridizing to both the Hb III and Hb IV genes located on 15.5 kb of DNA) and is in the range considered possible for DNA in the B-form. The average labeling density was 12.56 ± 6.07 IgG gold spheres / μm^2 ($n = 18$). The highest number of spheres recorded in any μm^2 area was 28. The level of significance of the labeling was very significant ($p < 0.001$) compared to the control area [a section containing both bands and interbands in division A1d, according Hägele's map of Ch. th. th. (1970)]. Unfortunately, the typically flaired nature of the telomeric region of chromosome III made it difficult to make a more precise determination of the extent of the labeling in either the band or interband regions. Additional labeling was also seen repeatedly to a group of bands and interbands in the same chromosome division. The exact reason for this has not yet been determined.

Immunofluorescence LM of a squash preparation and EM immunogold labeling of a SSP chromosome preparation of salivary gland chromosome II

Figure 42A depicts the hybridization site of the biotin-labeled haemoglobin IV gene to the salivary gland chromosome II of a Chironomus th. thummi squash preparation as detected by indirect immunofluorescence.

Figure 42B is a LM photo of a SSP chromosome preparation of chromosome II from a Chironomus th. thummi x Chironomus th. piger hybrid before in situ hybridization. Figure 42C is an electron micrograph of the area indicated in Figure 38B after in situ hybridization with biotinized haemoglobin IV gene probe and subsequential labeling of the hybridized site with 40 nm immunogold reagent. The labeling appears to cover both band and interband material. A portion of the interband material has apparently been spewed over and beyond the telomeric region as a result of the denaturation

and hybridization process. The average labeling density of was determined as 11.3 ± 6.7 IgG gold spheres / μm^2 ($n = 50$). The highest number of spheres recorded in any μm^2 area was 31. The level of significance was very significant ($p < 0.001$) in comparison to the control area [a section containing both bands and interbands in subdivisions A1g-i according to Hägele's map of the Ch. th. th. (1970)].

In spite of the flaired nature of the telomeric region, it was possible to observe through the higher resolution of the EM preparations, that even so-called "housekeeping" genes appear to occupy both interband and band areas.

The method which I have devised for transferring in situ hybridized and immunogold labeled SSP chromosomes onto grids for electron microscopy (Methods section 4.3.5.) has several advantages over the one described by Wu and Davidson (1981) as used by Kress (1985):

1) No lengthy pre-soaking in buffer solutions is necessary.

2) Only the chromosome intended for immediate transfer needs to be spotted with nitrocellulose, the others remain accessible, for example, for double labeling experiments.

3) The preparations are floated off on H_2O , completely eliminating the use of hydrofluoric acid which can destroy chromosomal structures, weaken the film material, and is, in general, hazardous to work with.

4) It works on a routine basis.

Another possible alternative was to carry out the hybridizations on chromosomes picked up on gold grids as has been done for metaphase chromosomes (Hutchison et al., 1982;

Fostel et al., 1984). In a number of attempts to do this, an extremely high film breakage was encountered and thus, loss of the preparations. This was due to the fact that the size of the slots needed to accommodate polytene chromosomes and the surface area which the film must span were much larger than those required for metaphase chromosomes. As a result, not only the film, but also the EM grids were much less stable than those used for metaphase chromosomes.

Immunogold labeling of thin-sectioned salivary gland polytene chromosomes has also been carried out (Hill et al., 1986), but here, once again, one has the same problems associated with the thin sectioning technique in general as noted in the Introduction.

At this point, I would also like to add a short comment on the staining of in situ hybridized and gold labeled SSP chromosomes intended for viewing with the electron microscope. I, generally, Giemsa stained the SSP chromosomes before coating them with nitrocellulose. Giemsa staining is one of the typical staining methods available for increasing the contrast of chromosomes viewed by light microscopy. As far as I am informed, no one has thought of using this for electron microscopy. I found in the case of the SSP chromosomes, that a better contrast could be more quickly achieved than with typical EM staining solutions such as uranyl acetate.

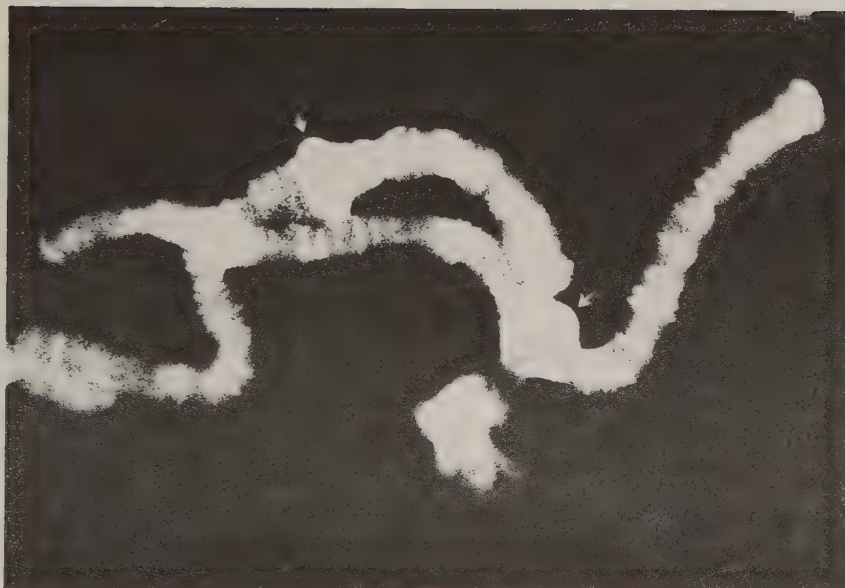


Figure 36. Indirect immunofluorescence detection of hybridized biotin-labeled "Cla-element" on squash preparations of salivary gland polytene chromosomes I and III of Chironomus th. thummi with rabbit anti-biotin primary antibodies and goat anti-rabbit FITC-conjugated secondary antibodies. Arrows indicate strongly labeled centromeric regions. 1,350x. (Source: Whitmore, unpublished)



Figure 37. Autoradiographic light microscopic (LM) detection of hybridized ^3H -labeled "Cla'-element" on a surface-spread polytene (SSP) chromosome preparation of salivary gland chromosome I of Chironomus th. piger. Giemsa stained. 1,100x. (Source: Whitmore, unpublished)

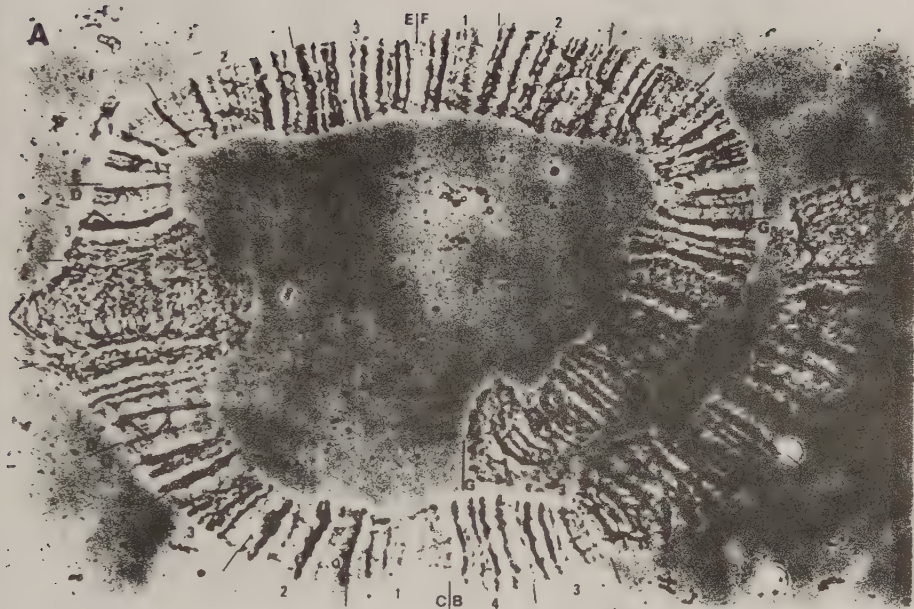


Figure 38A. Phase contrast light microscopic photo of a SSP chromosome preparation of *Chironomus thummi thummi* chromosome I before hybridization. Division sectioning according to Hägele (1970). 725x. (Source: Whitmore, unpublished)

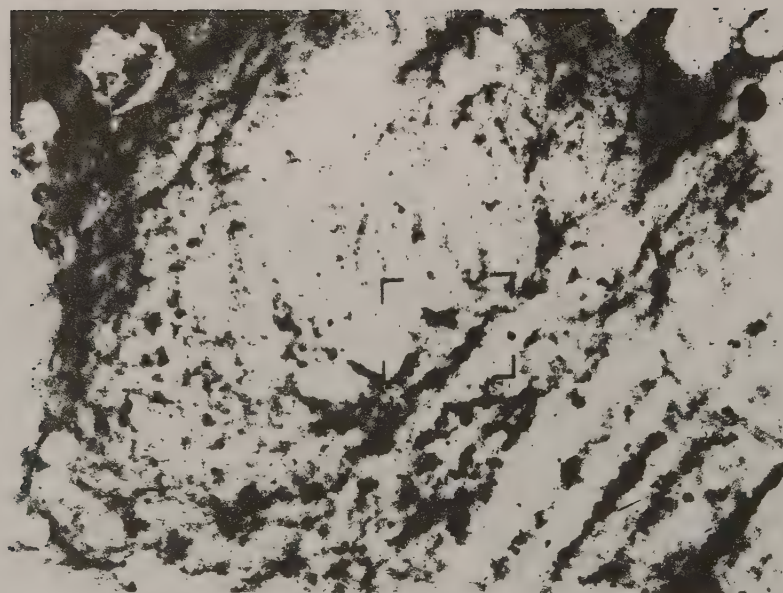


Figure 38B,C. (B) An electron micrograph of the area indicated by brackets in Figure 34A in the centromeric region after hybridization with biotin-labeled "C1a-element" and detection and subsequential labeling of the hybridized sites with rabbit anti-biotin primary antibodies and goat anti-rabbit 20 nm immunogold reagent. 3,200x. (C) Cut-out indicated by brackets in "B" at a higher magnification. 20,000x. (Source: Whitmore, unpublished)

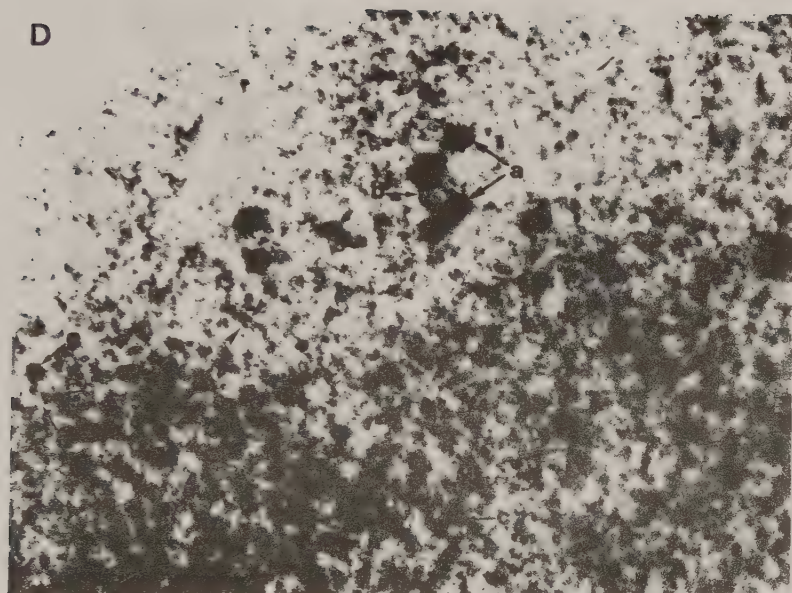


Figure 38D. A typical ultrastructural electron microscopic (EM) depiction of the state of the chromatin condensation at the sites of labeling depicted in Figures 38B, C. 200,000x. Indicated by arrows are (a) ca. 25nm diameter immunogold particles, (b) a thin DNP fiber of ca. 3.5 nm thickness with a beaded structure, (c) presumably, ca. $6 \times 10 - 11 \times 14$ nm nucleosome structures with very thin fibers attached, (d) examples of the very thin fibers with ca. 2 - 2.5 nm thickness, (e) a ca. 12.5 - 15 nm diameter ring or elliptical shaped structure which appears to be one subunit of an unit fiber, (f) ca. 5 - 6 nm thick DNP fibers running parallel to each other which seem to be composed of two finer ca. 2 - 2.5 nm fibers. (Source: Whitmore, unpublished)

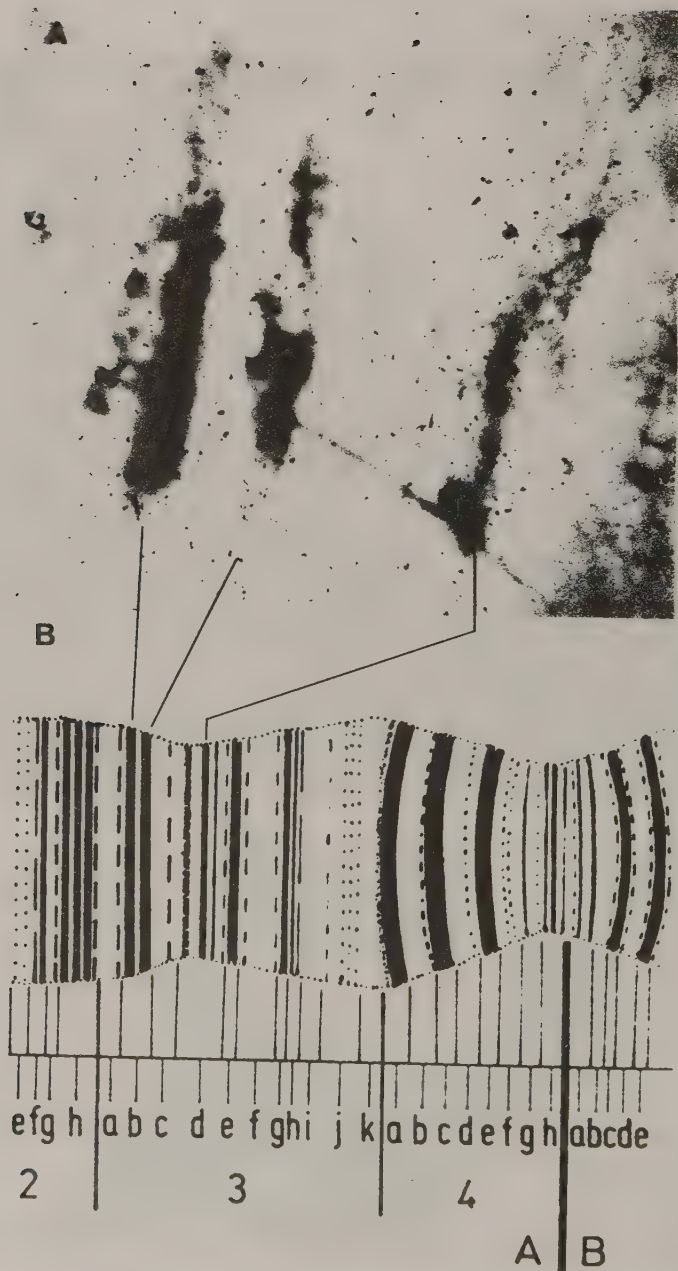


Figure 39A,B. (A) An electron micrograph from division A3 of a SSP chromosome preparation of Chironomus th. thummi chromosome I after hybridization with biotin-labeled "Claelement" and detection and subsequential labeling of the hybridized sites with rabbit anti-biotin primary antibodies and goat anti-rabbit 40 nm immunogold reagent. 5,520x. (B) The hand-drawn chromosome map of the respective section from Hägele (1970). (Source: Whitmore, unpublished)

C.

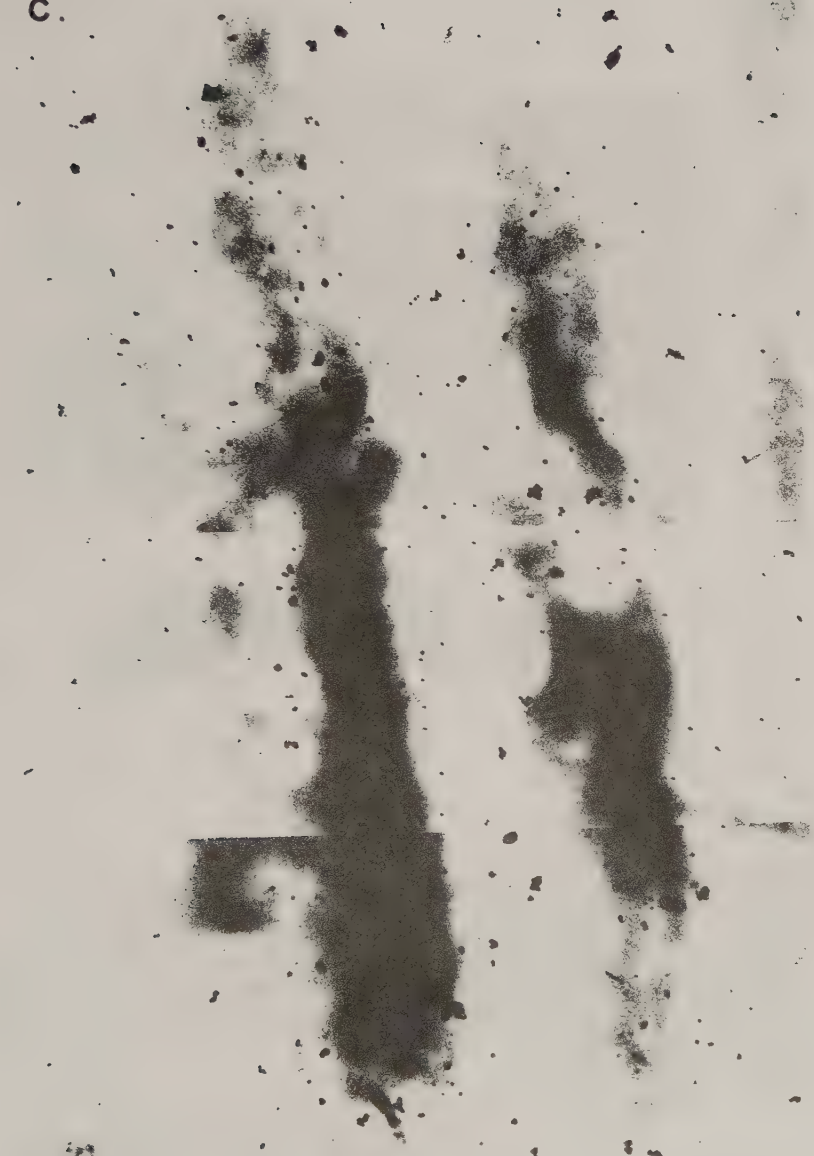


Figure 39C. Cut-out from Figure 38A at a higher magnification depicting subdivisions A3a and A3b. 12,000x. A number of gold conglomerates can be seen, i.e. groups of ca. 4 - 12 gold particles in close association. It was found that the number of such conglomerates could be reduced, however, through an additional centrifugation step as described in Methods section 4.3.4. (Source: Whitmore, unpublished)

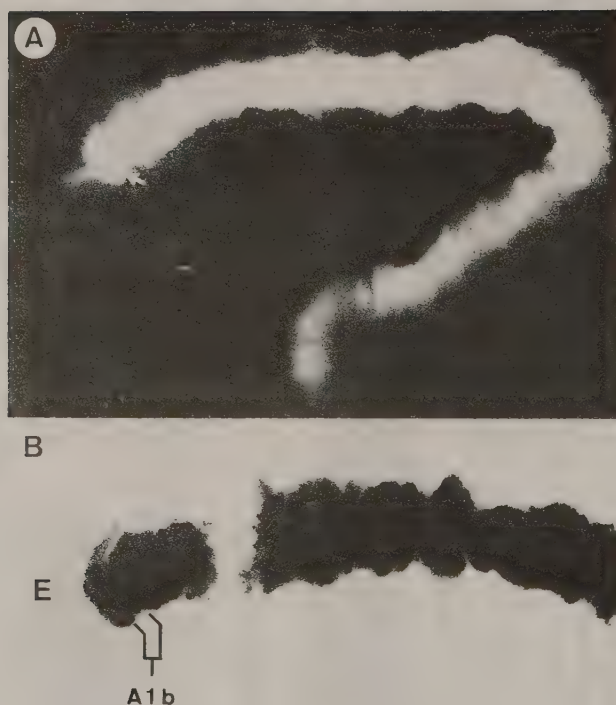


Figure 40. (A) Indirect immunofluorescence detection of the hybridized biotin-labeled haemoglobin IV DNA sequence on a squash preparation of salivary gland chromosome III of *Chironomus thummi thummi* with rabbit anti-biotin primary antibodies and goat anti-rabbit FITC-conjugated secondary antibodies. Arrow indicates site of labeling. 1,350x. (B) The identical chromosome after Giemsa staining. 2,700x. (Source: XI)



Figure 41. (A) Phase contrast light microscopical photo of a SSP chromosome preparation of chromosome III of Chironomus thummi thummi before being used for in situ hybridization. 800x. (B) An electron micrograph of the area indicated by brackets in (A) after hybridization with biotin-labeled haemoglobin IV gene probe and detection and subsequent labeling of the hybridized site with rabbit anti-biotin primary antibodies and goat anti-rabbit 20 and 40 nm immunogold reagents. Chromosome divisioning according to Hägele (1970). 7,700x. (Source: Whitmore, unpublished)

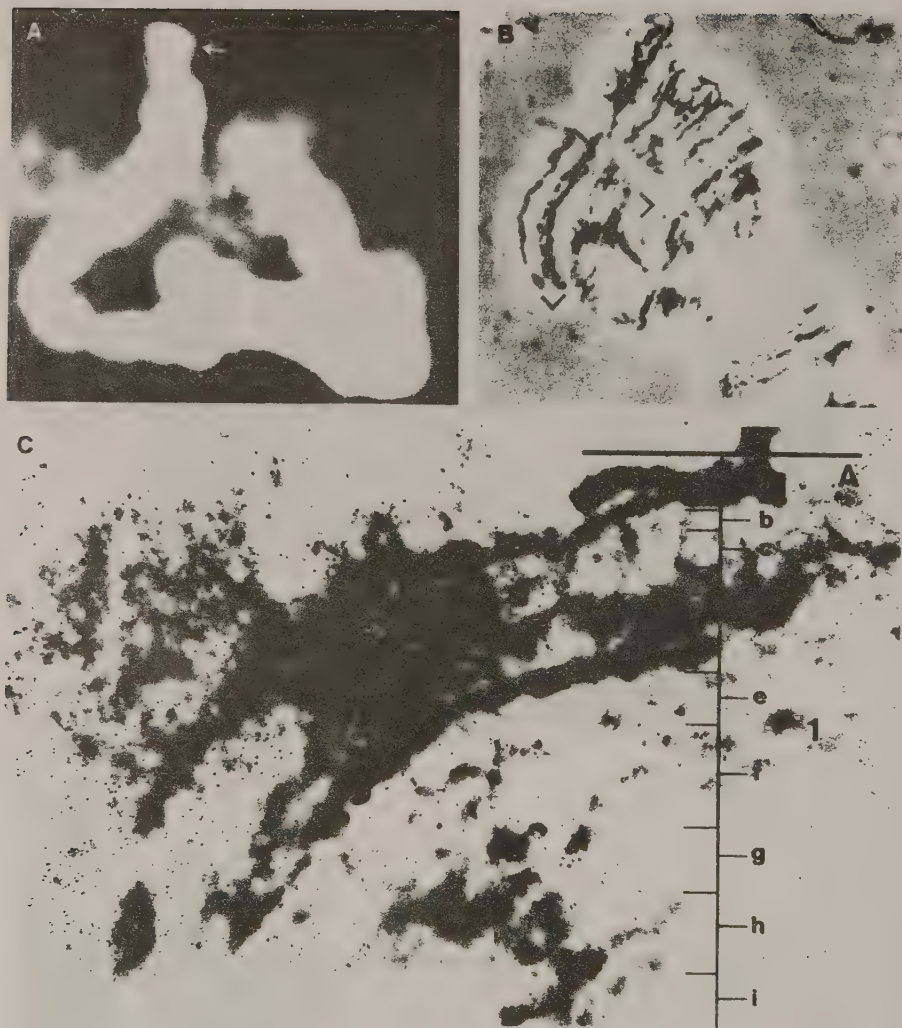


Figure 42. (A) Indirect immunofluorescence detection of the hybridized biotin-labeled haemoglobin IV DNA probe on a squash preparation of salivary gland chromosome II of *Chironomus th. thummi* with rabbit anti-biotin primary antibodies and goat anti-rabbit FITC-conjugated secondary antibodies. Arrow indicates labeled site. 1,350x. (B) Phase contrast light microscopic photo of a SSP chromosome preparation of chromosome II from a *Chironomus th. thummi* x *Chironomus th. piger* hybrid before in situ hybridization. 800x. (C) An electron micrograph of the area indicated by brackets in "B" after in situ hybridization with biotin-labeled haemoglobin IV gene probe and detection and subsequent labeling of the hybridized site with rabbit anti-biotin primary antibodies and goat anti-rabbit 40 nm immunogold reagent. 8,000x. (Source: Whitmore, unpublished)

5.6. Scanning electron microscopy (SEM) of surface-spread polytene (SSP) chromosomes without coating or conductive staining

(Source: Whitmore, unpublished)

Scanning electron microscopy (SEM) has been used in several studies to depict the ultrastructure of polytene chromosomes (e.g. Manning et al., 1975; Brady et al., 1977; Iino and Nagura, 1980; Kalisch and Jacob, 1983).

SEM has several advantages over transmission electron microscopy (TEM), for instance, 1) there is no need for thin sectioning, 2) the specimens can be mounted on a solid, nonbreakable surface, and 3) thicker objects yield a three-dimensional topographical view of the distribution of material.

Major disadvantages are 1) the, somewhat, lower resolution than TEM, and 2) the fact that the specimen to be viewed and the surface on which it is mounted must be conductive or made conductive to enable viewing. To accomplish this, most biological specimens are either coated with a layer (ca. 10 - 25 nm) of carbon or metal (reviewed by Echlin, 1978) or chemically treated through one of several osmium impregnation techniques (reviewed by Murphy, 1978). Conductive staining with osmium-TCH (thiocarbohydrazide), for example, adds only an estimated 3 - 4 nm layer to the object (Ip and Fischman, 1979), but is hampered by the occasional object contamination with osmium-TCH precipitate (Harrison et al., 1982). In both cases, the biological objects can now be viewed, but, due to the coatings, are no longer accessible for any further investigations (e.g. immunological, biochemical, hybridisation).

In this section, the technique described in section 4.6. of Methods is used for the first time to depict SSP chromo-

somes without coating or conductive staining by scanning electron microscopy.

SEM depictions of SSP chromosomes

Figure 43A1,2 is a SEM depiction of a Chironomus th. piger SSP chromosome III preparation which has been neither coated nor conductively stained. The salivary gland chromosome section (primarily division C3) is shown reversed to the hand-drawn chromosome maps of Ch. th. thummi (Keyl, 1957; Hägele, 1970), where the authors depicted the right chromosome arms on the left-hand side.

Figure 43A1 was taken using the normal polarity and the specimen holder was not tilted. It bears a strong resemblance to TEM chromosome depictions and in striking contrast to usual SEM depictions with coated specimens here the chromosome and cellular debris are color inverse to the background. In Figure 43A2 the polarity was reversed and here too, the chromosome and cellular debris are color inverse to the background. In both cases, the resolution of the chromosomal structures is good in comparison with TEM micrographs and the contrast sharp.

Figure 43B is a cutout from the area indicated by the arrow in Figure 43A1. At 10,000 x, the structural resolution is still good. The specimen table was fully tilted here and it is quite noticeable that the structures at the top of the picture are no longer as clear as those further down in the picture. In the interband section a rather heterogeneous mixture of particles and fibers can be seen ranging from ca. 10 - 60 nm in thickness.

In comparison to Hägele's (op. cit.) revised, hand-drawn chromosome map of Ch. th. th. (subdivisions C3g-i), an additional dotted band can be seen with globular particles of ca. 120 to 160 nm thickness (see arrow in Figure 43B). Fig-

ure 43C is the identical section as in 43B, but now sputtered with a 2 nm layer of gold.

The fact that the chromosome was already laying on a conductive gold surface apparently alleviates the necessity of a thicker layer. Here, the more typical SEM depiction is seen. The shapes of the chromatin fibers and globular particles are more accented than in Figure 43B. Already noticeable is, however, a filling in of small grooves or indentations as well as a covering over of extremely fine fibers and particles with even this thin gold coating. This indicates the importance of being able to observe non-coated specimens with SEM as shown in Figures 43A,B.

In Figure 43D, a compact Chironomus th. piger SSP chromosome III preparation is depicted by normal polarity and with the specimen holder fully tilted. The salivary gland chromosome is, like in Figures 43A and 43B, neither coated nor conductively stained. Here, due to the thickness of the chromosome, the three-dimensional effect of SEM is clearly evident. The ca. 1.0 - 1.2 μ m thick bands split near the edge of the chromosome into ca. 400 nm double or triple bands, respectively. The amorphous surface of the thick bands appears to be studded with 30 - 60 nm globular particles. Smaller bands seen in the vicinity of the larger ones are evident as globular particles ranging in size from ca. 50 - 200 nm, whereby the large 200 nm particles appear to consist of circular rings of approximately five 50 - 60 nm particles.

Using the technique introduced here (Methods section 4.6.), it was shown that the possibility exists of being able to view SSP chromosomes picked up on gold sputtered cover slips without coating or conductively staining the preparation. In comparison with a subsequently metal coated preparation, the resolution of the chromosomal structures was shown to be of similar quality at a magnification of 10,000x.

Chromosomes prepared in such a manner have several distinct advantages:

(1) They are still available for modern immunologically based chromosomal investigation techniques.

(2) The thickness of the chromosomal structures is not increased nor are smaller structures and indentations or grooves covered over and thus, obscured.

(3) The methodology used, produces a specimen which is color inverse to the background, yielding a high specimen/background contrast.

(4) For structural comparison purposes, the objects can be coated with as little as a 2 nm layer of gold.

(5) The procedure is of a rapid and relatively simple routine nature.

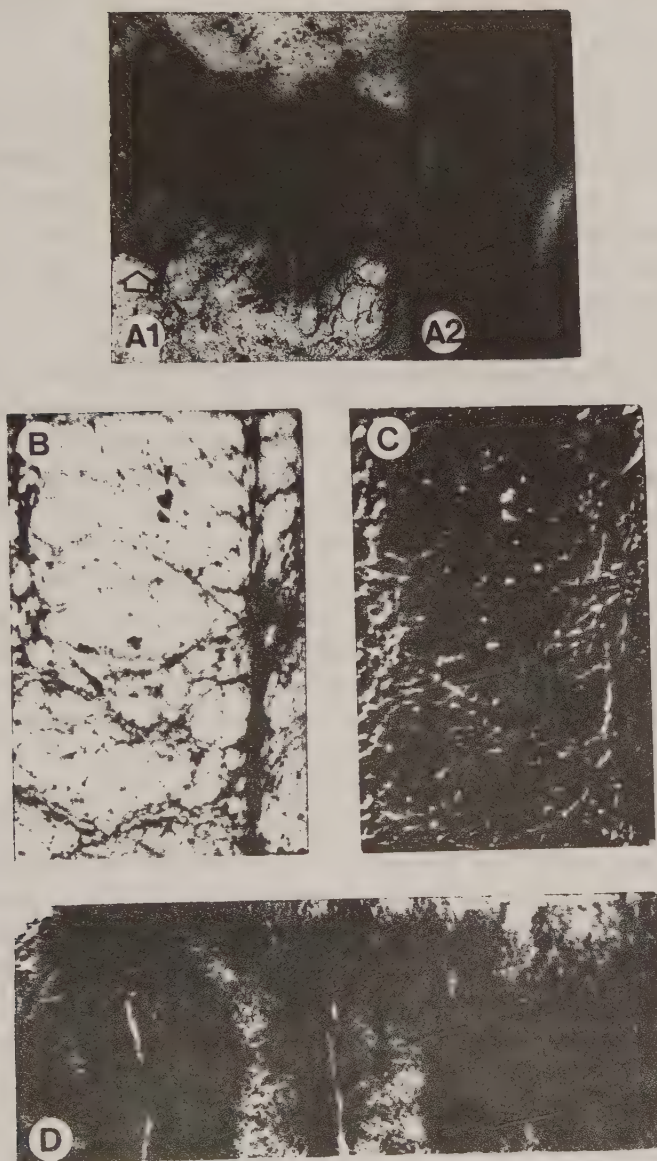


Figure 43. (A) Scanning electron microscopic (SEM) depiction of a *Chironomus th. piger* SSP chromosome III preparation, divisions C4 - C2. 1,000x. (A1) By normal polarity, without coating or conductive staining of the preparation. (A2) By reverse polarity and otherwise identical conditions as in A1. (B) Cut-out of the area indicated by brackets in "A1" at a higher magnification. 10,000x. (C) The identical chromosome section as in "B", but now sputtered with a 2 nm layer of gold. 10,000x. (D) A compact *Chironomus th. piger* SSP chromosome III preparation of division C1 depicted by SEM without coating or conductive staining of the specimen using normal polarity. 5,000x. (Source: Whitmore, unpublished)

5.7. The use of surface-spread polytene chromosomes for fluorescence microscopic analyses in Drosophila and Chironomus

(Sources: Based on XII and XIII)

In the past, immunofluorescence and fluorochrome studies using squash preparations of polytene chromosomes of various dipteran species have aided in the study of the general substructural organization of chromosomal bands and interbands (e.g. Howard et al., 1981; Nordheim et al., 1981; Gronemeyer et al., 1981; Lemeunier et al., 1982; Mortin and Sedat, 1982; Carmona et al., 1985; Rykowski et al., 1988). The fluorescence light microscopic analyses of the banding patterns in polytene chromosomes has been limited, however, by both the juxtaposition of the individual polytene structures and smaller chromosome bands with low contrast.

It was investigated in this section if SSP chromosomes could be useful in improving the fluorescence light microscopic resolution of banding patterns and other chromosomal characteristics which would thus enable a better investigation of polytene chromosomes in instances where the high resolution capability of electron microscopy is not available out of methodological or technical reasons.

Drosophila

The DNA-specific fluorochrome Hoechst 33258 (Weisblum and Haenssler, 1974) was used to stain Drosophila hydei SSP chromosome preparations. This bibenzimidole derivative has been used extensively in the past as a DNA-specific fluochrome in cytofluorometric investigations of metaphase chromosomes (e.g. Holmquist, 1975; Latt and Wohlleb, 1975; Wheeler and Altenberg, 1977; Singh and Gupta, 1982). Its use with polytene chromosomes has

been, however, rather limited (Holmquist, 1975; Lakhota and Mishra, 1980; Mortin and Sedat, 1982).

In this study, it was found that Hoechst 33258 could be used effectively on Drosophila SSP chromosome preparations, similar to corophosphine O (Nash and Plaut, 1965; Barr and Plaut, 1966), as a simple tool to help highlight intranucleolar DNA (nucleolar chromatin threads), intercalary chromosome regions "ectopic pairing" (Figure 44A) and puffed regions (Figure 44B) as well as for the depiction of the banding patterns (Figure 44C).

Figure 45 shows a direct comparison between D. hydei salivary gland chromosome 6 from (A) a squash preparation of good quality, (B) a fluorescence microscope photo of a SSP chromosome preparation, and (C1,2) two electron micrographs from SSP chromosomes which show the typical forms of chromosome 6. Here, the use of SSP chromosome preparations appears to improve the depiction of structural details over those seen in squash preparations.

Chironomus

Figure 46 shows a direct comparison of the banding pattern of polytene chromosomes of Chironomus thummi piger salivary glands from (A) a squash preparation of good quality, (B) an electron micrograph of a SSP chromosome, and (C) a photomicrograph of a fluorochrome stained SSP chromosome. The chromosomes shown have comparable degrees of polyteny. All three chromosomes are shown at the same magnification.

The fluorochrome stained chromosome (Figure 46C) reveals more structural details than the homologous chromosome region of the squash preparation (Figure 46A). There appears to be no sign of overstaining of the

prominent structures as is usually the case with squash preparations of polytene chromosomes. A comparison of Figures 46B and 46C shows that with SSP chromosomes a resolution can be obtained comparable to that of low power electron microscopy .

It was shown in this section through the use of surface-spread polytene chromosomes that in both Drosophila and Chironomus not only could an improvement be achieved in the depiction of structural details of the banding pattern in comparison to those seen in squash preparations, but that also structural peculiarities could be highlighted such as intranucleolar DNA (nucleolar chromatin threads) and intercalary chromosome regions "ectopic pairing".

The better resolution with the SSP chromosomes was the result of two factors. First, longitudinal spreading of the bands and interbands. Through this, it is possible to detect structures which are mostly hidden in juxtaposed sections of squash preparations. Second, well spread DNA material helps to prevent overstaining of even the most prominent bands, such as the centromeric band in C3 of the Chironomus preparation (see Figure 46C). The use of fluorochromes in squashed polytene chromosomes normally causes overstaining of the more prominent structures.

Finally, the comparison of the fluorescence photographs of the SSP chromosomes with the EM micrographs of SSP chromosomes (Figures 46B and 46C, and Figures 45B and 45C1-2) of the same regions showed a resolution of the polytene structures which was similar to that of low power electron microscopy.

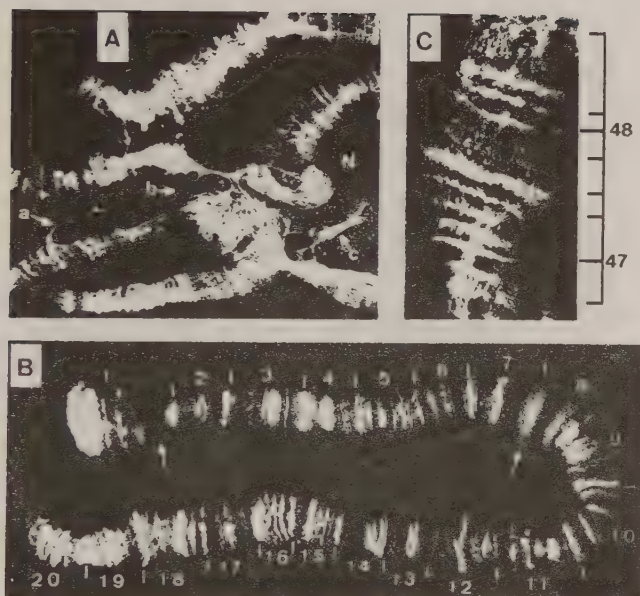


Figure 44. Photomicrographs of Hoechst 33258 fluorochrome stained SSP chromosomes in *Drosophila hydei*. (A) Chromocenter with nucleolus (N), arrows indicate (a) intercalary DNA "ectopic pairing", (b) proximal connections between chromosomes, and (c) intranucleolar DNA (nucleolar chromatin thread). 750x. (B) X chromosome, arrows point to two examples of puffed regions by 1d and 7d/8a. 550x. (C) Chromosome 2, region 46D - 48C, showing the band/interband pattern of a highly spread chromosome. 650x. (Source: XII)

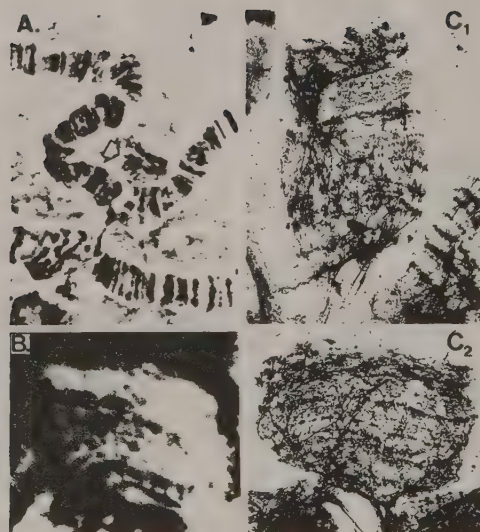


Figure 45. Drosophila hydei salivary gland chromosome 6. (A) Squash preparation, arrow indicates chromosome 6. Orcein staining. 1,600x. (B) Fluorescence microscope photo of a SSP chromosome preparation complexed with the DNA specific fluorochrome Hoechst 33258. 3,200x. (C1 - C2) Two electron micrographs of SSP chromosome preparations showing the typical forms of chromosome 6. 3,200x. (Source: XIV)

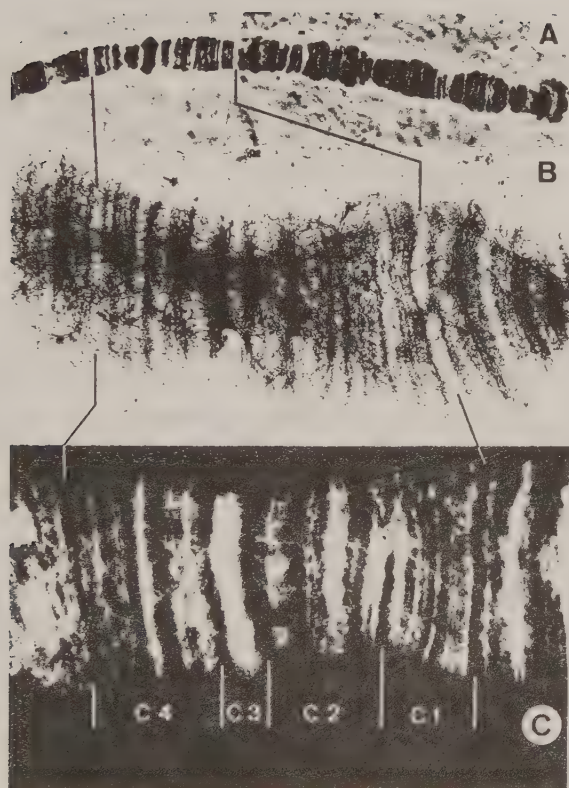


Figure 46. Banding pattern of polytene chromosomes of *Chironomus thummi piger* salivary glands. Centromeric region (C1 - C4) of chromosome II. Final magnification of all panels is 800x. (A) Light photomicrograph. Common squash preparation from a fourth instar larva. Orcein staining. (B) Electron micrograph, routine SSP chromosome preparation from a fourth instar larva after acid pretreatment and spreading. The electron micrograph was originally taken at 1,600x and 40 kV. (C) Fluorescence microscope photo of a SSP chromosome preparation complexed with Hoechst 33258. (Source: XIII)

5.8. A comparative analysis of the structural depiction of surface-spread polytene (SSP) chromosomes using differential interference contrast (DIC) microscopy with incident light and laser scanning

(Sources: Based on VIII and XVI)

For cytological mapping of polytene chromosomes, transmission light and electron microscopy have been used almost exclusively so far, because surface structure studies of native chromosomes have not been able to yield any detailed information concerning the banding patterns. This is the case even in scanning microscope analyses (Iino and Nagura, 1980). Using the surface-spread polytene (SSP) chromosome preparation technique, however, where chromosomes are spread laterally and longitudinally, more structural details are depicted in transmission light (e.g. Kalisch, 1982) and electron microscopy (e.g. Kalisch and Whitmore, 1983) than can be seen in well-extended squash preparations. Through the spreading process the SSP chromosomes are flattened enough so that individual bands become distinguishable as surface structures due to their supercoiled DNA (in comparison with the relatively uncoiled DNA of the interbands). It has been shown previously with scanning electron microscopy that the surface pattern of SSP chromosomes is identical with the one which can be seen using transmission electron microscopy (Kalisch and Jacob, 1983).

In this study, it was attempted to present light microscopically a more detailed photographic depiction of the band-interband pattern of polytene chromosomes from salivary glands than had been possible up to now with the usual routine technique of phase contrast photos of squash preparations. A major prerequisite was also the depiction of the chromosomes without using dye based contrast procedures in

order to be able to use them later for molecular-genetical investigations.

The initial investigation was carried out on a SSP preparation of Drosophila using a DIC microscope with incident light and later, as it was made available, the Zeiss "Laser Scan Microscope" (LSM) for a more comprehensive comparison using SSP chromosome preparations of Chironomus salivary glands.

Drosophila

Shown in Figure 47 is region 17D - 20D of the X chromosome from a late third instar larva of a D. hydei wild-type strain. In this figure the same SSP chromosome preparation is depicted with (A) incident- and (B) transmission light. In both cases, the patterns are identical, with the exception that in sections with lower degree of spreading, some additional structures (faint chromosome bands and tight double bands) can be seen with incident light (compare structures labeled in A and B).

Additional structures seen in (A) also coincide with the pattern seen by transmission electron microscopy. The chromosome map in (C) shows the pattern known so far from many transmission light microscopical studies on well-extended squash preparations.

The pattern given by this map cannot be depicted, however, from an individual squash preparation for several reasons: 1) Squash preparations are too thick, so that not all of the structural details of the pattern are in focus together. 2) The bands are often so tightly neighbored that the individual band can be distinguished light microscopically only after stretching the chromosome region. 3) The routine staining methods for chromosomes, which are primarily used for the contrast

of the thinner bands, over-stain at the same time groups of closely neighbored bands, especially the prominent ones.

The use of the SSP chromosome with DIC microscopy and incident light offers in contrast to squash preparations, a comparable pattern to the chromosome map with the additional advantage of a surface depiction which shows the distribution of chromosomal DNA in individual bands.

Chironomus

Figure 48a shows a region from salivary gland chromosome I of Chironomus thummi thummi. The orcein stained chromosome from a squash preparation was photographed using phase contrast and a green filter on a conventional light microscope.

Figure 48b shows the homologous chromosome region of a SSP chromosome photographed with differential interference contrast on the Laser Scan Microscope (LSM). Routine squash preparations like that in Figure 48a yield light microscopically with all previously used methods and inspite of being stained, only a reduced pattern photographically when compared to the large number of bands depicted in the chromosome map in Figure 48c.

The pattern of the chromosome map was determined after time-consuming microscopic investigations on a large number of selected chromosome squash preparations (Hägele, 1970). Several of the very fine bands in the chromosome map are at the limits of light microscopic resolution and in addition, have been identified in all of the previous light microscopic analyses only a few times (Hägele, personal communication). The existence of these bands has been, however, adequately proven through

electron microscopic investigations on thin-sectioned chromosomes (Kiknadze et al., 1976) and on SSP chromosome preparations (Kalsich et al., 1984, and unpublished data).

The depiction of the SSP chromosome in Figure 48b is shown using the same enlargement factor as in Figure 48a. Through the spreading technique the longitudinal distances between the bands are increased by approximately three-fold in comparison to the squash preparation. In the lateral direction the chromosome shows an approximate six-fold degree of spreading in comparison to the squash preparation. The spreading process is combined with a very strong flattening of the chromosome. Thereby, the band structures, which are more condensed than the interbands, appear as three-dimensional surface structures. The differential interference contrast photo in Figure 44b shows for the first time light microscopically these structures.

In Figure 48b not all of the structures drawn in the chromosome map are clearly visible (for example, a few of the very fine bands on the border between the subdivisions F3 and F4). The technique allows nevertheless more details to be recognized on the whole as in the squash preparation in Figure 48a. Thereby is to be considered that altogether three techniques led to the improved depiction: 1) SSP chromosome preparation versus squash preparation. 2) Laser Scan Microscope versus conventional light microscope. 3) Differential interference contrast versus phase contrast.

In order to roughly judge the contribution of the individual techniques to the improved depiction of the pattern details, a SSP chromosome was photographed stepwise with the various techniques. Figure 49a shows a phase contrast photo of a strongly spread, unstained salivary gland chromosome I of division DE from Chiron-

omus th. piger with a large number of thin bands with low contrast. A light microscopic identification of fine individual bands is difficult due to the spreading of the unstained structures and can be accomplished only after over-staining the chromosomes (Kalisch, 1982) or after the use of fluorochromes (Whitmore and Kalisch, 1984).

The use of phase contrast with Laser Scan Microscope in Figure 49b shows a better contrast, but no visible improvement in the resolution of pattern details. In Figure 49c incident light with differential contrast was used with a conventional light microscope.

Surprisingly, the identification of the fine bands is better than in the phase contrast depictions of Figures 49a and 49b. Improvement in the resolution of band - interband details through the use of DIC and incident light in comparison with phase contrast is, however, a special effect of the SSP chromosome preparation technique itself. Unspread polytene chromosomes photographed with DIC and incident light, although impressive in their general appearance, are rather unattractive in as far as the depiction of pattern details is concerned because of the tight juxtaposition of polytene structures (Hägele, 1980).

In Figure 49d, incident light and DIC were used with the LSM. The chromosome depicted shows, in general, a better contrast than Figure 49c. However, by this magnification, it is difficult to judge whether or not the use of the LSM also yields an improvement in the resolution of pattern details. For this analysis, region B4d - C2d of SSP chromosome I in Chironomus th. piger was photographed under comparable conditions with incident light, DIC and with an Epiplan 80/0.95 Pol objective using the conventional light microscope (Figure 50a) and the LSM (Figure 50b).

The comparison shows that not only a higher contrast, but also more structural details are depictable with the Laser Scan Microscope in both the prominent chromosome bands and the interbands. Additional polytene structures are also visible (especially in the interbands). The number and position of these structures coincide very well with the pattern of the homologous region in the chromosome map of the subspecies Chironomus th. thummi (Hägele, 1970) and with electron microscopic studies (unpublished data).

An improved depiction of the band-interband pattern in Chironomus salivary gland chromosomes was thus achieved through the additive effect from the use of the Laser Scan Microscope, the depiction of the surface structures with the differential interference contrast and the use of SSP chromosome preparations. The SSP chromosome preparation technique itself allowed, in comparison to squash preparations, tightly neighbored structures to be spread strongly enough so that these could be depicted individually.

In this section, it was shown that incident light microscopy together with differential interference contrast (DIC) could be used for a detailed pattern analysis of SSP chromosomes in Drosophila and that the resolution of incident light microscopy could be further improved through the use of the Zeiss Laser Scan Microscope as was shown with SSP chromosomes of Chironomus. The Laser Scan Microscope study was not intended to represent the entire modes of operation possible with this microscope (see Wilke et al., 1983), but to show its special use in the incident light mode. So far, only one comparable study exists where use was made, however, of the transmission light mode of the confocal scanning microscope of Brakenhoff (1979) and Brakenhoff et al. (1979) with squash preparations of polytene chromosomes in Drosophila hydei (Grond et al., 1982).

Generally, the use of differential interference contrast avoids the disturbing halation effect seen around individual chromosome structures in phase contrast microscopic analyses. This halation effect has played, historically, an important role in chromosome structure research because through it double bands can be imitated (e.g. see Sorsa et al., 1983). In this connection, however, a critical note should be added in regards to the incident light DIC used here for the depiction of the chromosome surface. Artificial bands might appear in particular settings of the Normarski prism if there are positions of equal inclination along the slope of a band.

The use of the combination of incident light-, differential interference contrast-, and laser scanning microscopy together with SSP chromosome preparations was shown to have the advantage in comparison to the light microscopic techniques used so far of: 1) the use of unstained SSP chromosomes, 2) being able to depict the distribution of total chromosomal DNA in individual bands, and 3) yielding additional information about pattern details which could be documented photographically.



Figure 47. Region 17D - 20D of a SSP chromosome preparation of the X chromosome from a late third instar larva of a *Drosophila hydei* wildtype strain (Sao Paulo). (A) Incident light, differential interference contrast (Epiplan 80/0.95 Pol objective). (B) Transmission light, phase contrast (Planapo 63/1.4 Ph3 oil objective). (C) Chromosome map of Berendes (1963) based on transmission light microscopic analyses of many squash preparations. Bar represents 20 μ m. (Source: XVI)

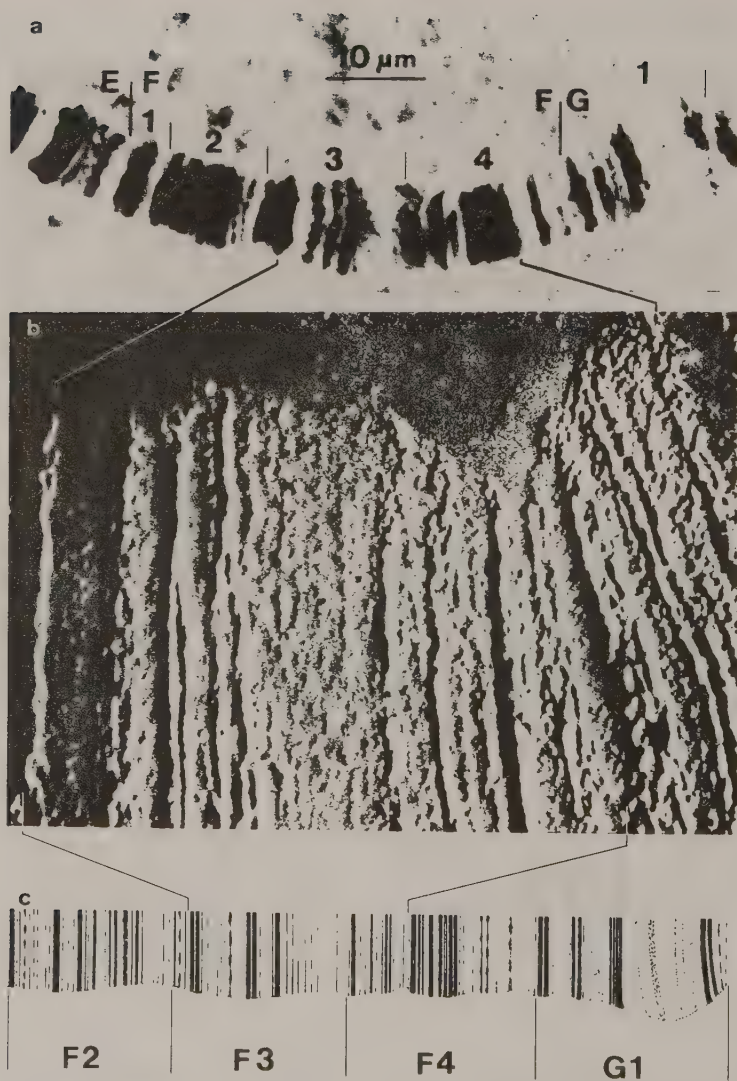


Figure 48. Band/interband pattern of division F in the left arm of salivary gland chromosome I in *Chironomus th. thummi*. (a) Squash preparation, Orcein staining, Planapo 63/1.4 Ph3 objective, 1.4 oil condensor and green filter, 1,800x. (b) Cut-out of an unstained surface-spread polytene (SSP) chromosome as seen in the Laser Scan Microscope with differential interference contrast and incident light, Epiplan 80/0.95 Pol objective. Same magnification in "a" and "b". (c) Cut-out of the hand-drawn chromosome map from Hägele (1970) based on light microscopic analyses of many selected, well-extended chromosomes in squash preparations. (Source: VIII)

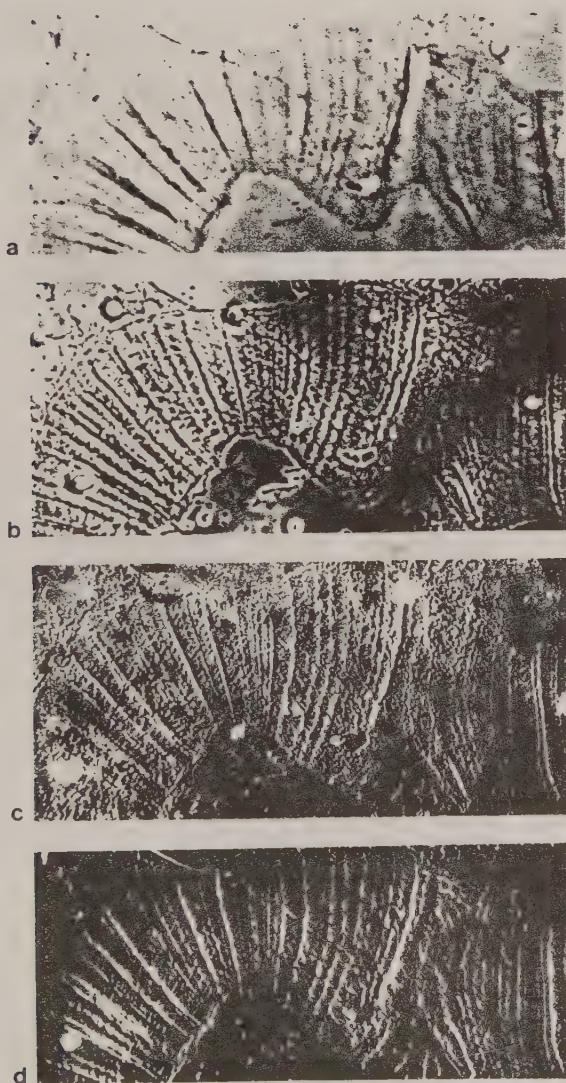


Figure 49. Band/interband pattern of the centromeric region (division DE) in the salivary gland chromosome I in *Chironomus th. piger*. SSP chromosome preparation, unstained. (a) Phase contrast, conventional light microscope, Planapo 40/0.95 Ph3 objective, 1.4 oil condensor and green filter. (b) Phase contrast, Laser Scan Microscope (LSM), Planapo 40/0.95 Ph3 objective, 1.4 oil condensor and green filter. (c) Incident light, differential interference contrast (DIC), conventional light microscope, Epiplan 80/0.95 Pol objective. (d) Incident light, DIC, LSM, Epiplan 40/0.85 Pol objective. 1,000x. (Source: VIII)



Figure 50. Band/interband pattern of region B4d - C2d in SSP chromosome I of Chironomus thummi piger. For both depictions of the same specimen incident light and differential interference contrast were used (Epiplan 80/0.95 Pol objective). (a) Conventional light microscope. (b) Laser Scan Microscope, 1,500X. (Source: VIII)

6. DISCUSSION

6.1. Introductory remarks

The preceeding results entailed a comparative compilation of the previously and newly available methods and techniques for the cytological depiction and analysis of polytene chromosome structure using the surface-spread polytene (SSP) chromosome preparation technique including those developed by myself. Thereby, special consideration was given to the additive effect of various light- and electron microscopic preparatory methods.

A similar, comparative and comprehensive analysis of this depth does not exist for any of the preparation techniques of polytene chromosomes known up to now. The new findings derived from this methodological analysis are dealt with in considering the applicability of the techniques described for investigating specialized questions concerning the cytological and molecular-genetical analysis of polytene chromosomes (e.g. the use of SSP chromosomes for high resolution studies involving the relationship between chromosome structure and function using fluorescence LM, laser scanning microscopy (LSM) with differential interference contrast (DIC), transmission electron microscopy (TEM) and scanning electron microscopy (SEM), LM autoradiographic- and EM immunogold labeled DNA hybridizations).

In addition to the methodological part of the study, through a combined use of the techniques developed and the SSP chromosome preparation technique the following points could be considered experimentally at the EM level (some extensively, others to a limited extent):

- The accuracy of LM determinations of band numbers.
- The theoretical coding potentials of the bands and interbands in SSP chromosomes.

- The intertissue constancy of the banding pattern of dipteran polytene chromosomes and the extent to which tissue specific variations really occur.
- The structural boundaries of transcriptionally active genes.
- The relationship between chromosome structure and the labeling of in situ hybridized polytene chromosomes at the LM and EM level.

The evaluation of the individual results based on the various methodology used and the data from the literature have already been discussed in detail from myself in the Results and/or in the respective publications. For that reason, the following discussion will be limited in this respect to a comparison and appraisal of the methods and techniques for the analysis of banding patterns, the construction of chromosome maps and the significance of labeling seen by in situ hybridization studies in relationship to chromosome structure at the DNA level.

Possible areas of future biological use will be discussed at the conclusion.

6.2. Concerning the methodology

6.2.1. Preparation techniques and the photographic depiction of band-interband patterns

During the some five decades of polytene chromosome research, the technique of squash preparation has been used for most of the routine investigations in light microscopy. The mechanical flattening of the more or less cylindrically shaped polytene chromosomes by the process of squashing has, however, several disadvantages for cytological analyses and for the photographic depiction of the chromosomes:

1) Generally, squash preparations are not flattened enough to get all the structural details of a larger chromosome region in focus. 2) Adjacent chromosome bands can become "fused" by the squashing process, leading to a reduced pattern (Kalisch et al., 1984). 3) Squash preparations are optically too dense to obtain a detailed EM depiction of the pattern.

As mentioned in the Introduction, a breakthrough in the analysis of the band-interband pattern of polytene chromosomes has been the use of the squash and thin-sectioning method for EM mapping (Sorsa and Sorsa, 1967). Despite its well-known advantages, this technique has four methodological disadvantages that have hampered the general use of this technique: 1) Thin-sectioning of polytene chromosomes is too time-consuming in routine work, especially, if an entire chromosome is to be depicted. 2) The analysis of immediately adjacent chromosome bands depends upon well-extended chromosome sections, which appear more or less by chance. 3) There is usually only a short and partial section of the entire chromosome available in a single preparation. Due to this, the total amount of DNA present in individual bands and interbands can only be estimated. 4) Some of the very fine bands may go unnoticed which have been seen in thin-section studies on native salivary gland chromosomes (Mott and Hill, 1986).

A technique of chromosome stretching by micromanipulation has been used to provide a better separation of most chromosome bands before the squashing process (Ananiev and Barsky, 1982). However, although this technique has the advantage of getting depictions of well separated bands and interbands even in Drosophila hydei, the mechanical stretching decreases the diameter of the chromosomes and does not improve the LM depiction of thinner bands [in comparison with the total number of bands registered in the chromosome maps of Berendes (1963)].

The mediums used for isolating and fixing chromosomes also play a role in the preservation and depiction of chromosomal structures. Ringer solution, which has been the standard solution used in the preparation of salivary glands for cytological studies involving polytene chromosomes, was shown by Kalisch and Whitmore (1983) to have an adverse effect on the spreading of polytene chromosome structures. In that EM study of Chironomus tepperi, the replacement of Ringer through pre-treatment solution (see Methods section 4.1., subscript 2) led to a ca. 150% increase in the number of newly recognizable bands in SSP chromosome preparations over SSP chromosomes isolated in Ringer. Others, have also previously attempted either to replace Ringer with another physiological solution (Mott et al., 1980), or to fix the glands initially (Skaer, 1974). The detrimental effects of the acetic acid treatment used in the classical squash preparation technique and its extraction of chromosomal proteins have been noted as well for quite some time (e.g. Beermann and Bahr, 1954; Goodwin et al., 1978; Saura and Sorsa, 1979; Hill et al., 1984).

The attempt to prepare polytene chromosomes as described in Methods section 4.1., started with the consideration that a spreading process on a aqueous surface could be used as a reliable method for a smooth transitional chromosome preparation and a photographic depiction without changing details of the pattern. Further advantages and disadvantages of the SSP chromosome technique in comparison with the methods mentioned above have been discussed earlier (Kalisch and Whitmore, 1983; Kalisch et al., 1985).

6.2.2. Light microscopic (LM) versus electron microscopic (EM) methodology - advantages and disadvantages in the study of polytene chromosomes

With the number of methodological possibilities used just alone in this study to investigate and observe polytene chromosomes, it is inevitable that questions arise such as:

Just when do I use this technique or that one? What are the advantages or disadvantages of the one technique in comparison to the others? When is the resolution of the one sufficient to fulfill my purposes and when is it necessary to turn to a method which offers a higher resolution? Is a combined use of several methods of an advantage?

These questions can be best answered in considering the systematic approaches taken in attacking, on the one hand, cyto-genetical problems (e.g. chromosome mapping or comparative chromosomal banding pattern analyses) or, on the other, molecular cyto-genetical problems (e.g. the localization of in situ hybridized DNA probes). Whereas, in the first case one is primarily concerned with the optimal depiction of the chromosomal structure (e.g. through high resolution microscopy or chromatin staining), in the latter, an additional problem is involved in having to retain the chromatin in an active state (e.g. the ability to be hybridized with DNA probes and also to be receptive for antigen/antibody reactions).

In both the first and the second category, the usual procedure was to begin with routine squash preparations at the LM level for the purpose of "familiarization" with already published chromosome maps or photo material. An advantage here is also the large number of chromosomes (genomes) per slide in comparison to SSP chromosome preparations. As the next step, SSP chromosomes were observed at the LM level either Orcein or Giemsa stained to correlate the chromosomal appearance between those seen in squash preparations and those in surface-spread preparations. By well spread SSP chromosomes it was found, however, that fluorochrome stained preparations (see Results section 5.2.) were more beneficial in depicting the chromosomal structures and peculiarities (e.g. banding patterns and ectopic pairing). The LM studies served then as a basis for the TEM investigations involving fine chromosome mapping.

In the second category (involving in situ hybridizations), one is confronted with the problem that by chromosomal denaturation much of structural details (e.g. a clear banding pattern) are lost. It is necessary, therefore, to obtain a highly resolved picture of the chromosome(s) before the denaturation process and this without being able to stain the chromosomes in any manner. The use of differential interference contrast (DIC) with the Laser Scan Microscope appears to be the best possibility to achieve this (see Results section 5.8.). It would be of interest in the future to use that combination in connection with preparations intended to be viewed with the TEM after the hybridization procedures. A possible future alternative is the use of SEM with SSP chromosome preparations picked up on gold sputtered cover slips. Although lower in resolution than the TEM, this methodology is of importance in helping to obtain a three-dimensional topographical conception of the structures involved and deserves, therefore, further investigating and refining. Also, in terms of magnification, the SEM picks up and goes several magnitudes beyond that what the Laser Scanning Microscope is capable of obtaining. It should also be noted that the SEM which I used was a relatively old model and that newer ones are capable of a considerably higher degree of resolution.

6.2.3. Photo maps versus chromosome maps

Independent of the technique of chromosome preparation used, individual micrographs (even of SSP chromosomes) do not always depict all of the structural details of a band-interband pattern in longer chromosome regions. This is not only true for Drosophila species, but also, to a certain extent, for Chironomus species. Generally, the number of chromosome bands and interbands which can be analyzed in individual polytene chromosome preparations may differ not only as a result of the degree of polyteny, the extension of the chromosomes, and the developmental stage specific

variations, but also from the technique used for the preparation and analysis. In the band-interband pattern analyses of polytene chromosomes, the presentation of cytological details has, thus, been an important problem. This has been resolved so far by hand-drawn chromosome maps stemming from a large number of cytological studies. Such maps have been constructed for many species of the Diptera with polytene chromosomes suitable for investigating (e.g. Beermann, 1952; Keyl, 1957; Berendes, 1963, 1971; Gabrusewycz-Garcia, 1971; Sorsa et al., 1984; Roberts and MacPhail, 1985; Staiber and Behnke, 1985). There have been, however, many cytological studies in which only parts of the polytene chromosomes have been analyzed or even revised. As a consequence, up to date chromosome maps which include all the cytological data previously published are almost nonexistent. Therefore, a computerized system to store cytological data and to draw computerized chromosome maps on the basis of these data as the one described in Methods section 4.2. and used in the mapping of the salivary gland polytene chromosomes X, 4, and 6 in Drosophila hydei in section 5.3. of Results, could be of help in solving this problem.

The construction of chromosome maps does, by no means, eliminate the need for good photo maps. In fact, the majority of investigators have great difficulties in relating the structures depicted in chromosome maps with those of actual chromosome preparations. The major reason behind this is fairly obvious, chromosome maps represent an ideal case and not what one is generally confronted with when looking through the microscope. In sections 5.2. and 5.4. of Results it was clearly demonstrated that the SSP chromosome preparation technique is well suited for the preparation of EM photo maps of entire genomes not only for the highly polytene salivary gland chromosomes of Drosophila and Chironomus, but also for the much lower polytene Malpighian tubule chromosomes of Chironomus thummi piger.

As indicated in Results section 5.3., the EM analysis of SSP chromosome preparation has, in general, increased the total number of bands registered for the salivary gland polytene chromosomes of *D. hydei* anywhere from 34 - 100% by revealing additional bands which have not been detected in LM analysis of chromosome squash preparations. Thus, the new reference maps presented can offer a more precise basis for comparable pattern analyses in different tissues and for gene localizations at the EM level.

6.3. Concerning the results

6.3.1. Interband lengths

Quantitative evaluations of the interband lengths in polytene chromosomes have been used to calculate whether or not they are long enough to accomodate eukaryotic genes. So far, the cytological results are contradictory. There are three main methodological reasons for this: 1) The tight juxtaposition of polytene structures even in well-extended or stretched chromosome squash preparations. 2) The small number of selected interbands measured. 3) Differences in the extent of the DNA packing ratio determined for the interbands, which has been estimated through densitometry or from the diameter of interband fibers (Laird, 1980; Laird et al., 1980; Sass, 1980; Alanen, 1981; Sorsa, 1982c, 1983).

An improved evaluation requires in part: 1) An analysis of all or at least the majority of the interbands for calculating average data. 2) A spreading technique, which elongates the axial length of a chromosome without rough mechanical stretching of chromosomal squash preparations, i.e. without changing the pattern. 3) A spreading technique which results in total extension of the DNA in all of the interbands. It has been shown that the SSP chromosome technique fulfills the first two prerequisites but gives no evidence that the DNA is completely extended in the inter-

bands. On the contrary, the fact that the total length of a SSP chromosome may vary dependent on the degree of spreading supports the finding of nucleosome structures in the interbands by Ananiev and Barsky (1985) as well as the calculations of the average DNA packing ratio in the interbands of SSP chromosomes by Kress et al. (1985).

A nucleosome depletion of the acid pre-treated SSP chromosomes during the spreading process may be possible, but the preliminary measurements of the size of the interband structures by TEM and SEM (Results sections 5.1. and 5.6.) seem to indicate that this is not the case. Also, as far as the unfolding of the chromatin by the 4 M urea is concerned, it has to be assumed that this procedure does not result in a complete stretching of the DNA, which can be only achieved in more than 6 M urea (Nielsen et al., 1985). Sorsa (1983) has stated that there is no support for the existence of nucleosome helices in the interbands after different fixations and differential stretching of the polytene chromosomes. However, in SSP chromosomes the average interband length is 50% longer than Sorsa's interbands calculated from squash preparations in Drosophila melanogaster (Sorsa et al., 1984). The argument that band material is pulled into the interband during the SSP chromosome preparation can be excluded by pattern analyses in chromosomes which showed different degrees of longitudinal spreading (Kalisch and Whitmore, 1983).

Concerning the actual lengths of the interbands and their coding potentials, it was shown that in SSP chromosomes, cytological values such as band diameter and width, interband length, interband and puff outlines, and radius of curved bands could be measured and digitized even for entire chromosomes (e.g. Results section 5.3.). The time-consuming technique of band by band digitizing was used, instead of a computerized pattern scanning, for greater accuracy in the interpretation of individual polytene structures. Through the combination of the two techniques, SSP

chromosome preparation and computerized plotting of chromosome maps, it was possible to compare the lengths of interbands. A discussion about whether or not the average interband is long enough to accommodate an average eukaryotic gene is difficult considering the small amount of molecular data concerning gene length at the DNA level, i.e. including the leaders, trailers, introns, and flanking landscapes. Table 13 (see Appendix) lists examples of cloned genes in Drosophila melanogaster and the lengths of their transcripts. It can be seen here that processed transcripts average around 1.0 kb - 1.7 kb. Depending upon the actual lengths of the nontranscribed sequences and sequences eliminated in processing a number of genes could possibly fit into the range of the average interband length found in Drosophila hydei for chromosome 4 (0.97 kb - 3.3 kb) and the X chromosome (1.1 kb - 3.8 kb) as well as in part for the short dot chromosome 6 (0.5 kb - 1.8 kb).

6.3.2. Cytological peculiarities

In Chironomus, SSP chromosomes from the salivary glands can be usually found without superpositions and with a comparable degree of spreading in all or at least in most of the divisions of the entire chromosomes. This spreading behavior cannot be expected in SSP chromosome preparations of Drosophila hydei because of the lower degree of polyteny and the characteristic end-to-end chromosome adhesions in this species (Berendes, 1963; Berendes and Meyer, 1968). Furthermore, the telomere fusion of the autosomes not only effects the spreading of the chromosome sections involved, but also usually interacts with the spreading process of the rest of the chromosomes.

For the identification of SSP chromosome sections, constrictions are useful cytological landmarks, and their biological meaning has been the object of considerable interest in the literature. Usually, polytene chromosome constrictions in squash preparations are characterized ac-

according to different peculiarities: ectopic pairing points, weak points, underreplicated chromosome sections, and late replicating polytene structures (Kalisch and Hägele, 1976; Zhimulev and Kulichkov, 1977). The differences found between SSP chromosome preparations and squash preparations (Results section 5.2., Table 2) are of interest inasmuch as the cytological identification and biological meaning of these sections are concerned and will require further investigation.

Also, in reviewing the almost 50 years of literature concerning comparative studies of naturally occurring polytene chromosomes from different tissues, differences in the presence or absence of weak points or intercalary DNA (ectopic pairing) have gone virtually unmentioned. This could be for a number of reasons: 1) There were no such differences in the tissues studied. 2) The differences were present, but felt of little importance, so they were not reported. 3) The limited resolution of the LM studies on chromosomes much lower in polyteny than those of the salivary glands did not permit accurate evaluations of this aspect.

Through the use SSP chromosome preparations combined with the higher resolution of the electron microscope, it was possible, for the first time, to make a comprehensive analysis of the differences in the physical appearance of intercalary heterochromatin in chromosomes of different tissues. The EM photo maps shown in Results section 5.4. of the Chironomus th. piger salivary gland chromosomes indicate that the expression of underreplication through noticeable weak points, ectopic pairing, and constrictions may depend largely upon simply the level of polyteny. The lower polytenized Malpighian tubule chromosomes showed typical signs of intercalary heterochromatin, whereas the higher polytenized salivary gland chromosomes did not. This may mean that the higher the degree of polyteny, the more rigid and stable the overall chromosome structure may

become and thus less likely to display sections of underreplication.

6.3.3. The banding pattern and its genetic significance

Most of the studies involved in seeking to determine the genetic significance of the banding pattern can be divided into several major categories:

(A) Investigations of the constancy and variation of the banding pattern between 1) polytene chromosomes of the same tissue and species, but under differing physiological conditions (e.g. Zhimulev et al., 1976; Ilinskaya and Maximova, 1976; Zhimulev and Belyaeva, 1977; Belyanina, 1977; Ilinskaya and Maximova, 1978), 2) polytene chromosomes of different tissues of somatic origin (e.g. Kosswig and Shengün, 1947; Shengün and Kosswig, 1948; Slizynski, 1950; Beermann, 1952; Pavan and Breuer, 1952; Sengün, 1954), and 3) chromosomes of different tissues and origin, i.e. somatic and germ-line mutants (Ribbert, 1979; Redfern, 1981a,b; Heino, 1989).

(B) Investigations of the variation in the puffing pattern between 1) polytene chromosomes of different tissues (e.g. Holden and Ashburner, 1978; Gupta and Singh, 1983; Singh and Gupta, 1985; Staiber and Behnke, 1985; Hochstrasser, 1987), or 2) of the same tissue, but at different developmental stages (e.g. Berendes, 1966; Roberts and MacPhail, 1985).

(C) Investigations of the transcriptional activity or possibility thereof in the band-interband structures through 1) the location of RNA synthesis through uridin label (e.g. Zhimulev and Belyaeva, 1975; Kerkis et al., 1977; Semeshin et al., 1979), by DNA:RNA hybrids (Vlassova et al., 1985), as well

as the mapping of transcription sites for poly-(A) RNA by in situ hybridization with cDNA (Kress et al., 1985), 2) the location of RNA polymerase B in the interbands (e.g. Jamrich et al., 1977; Sass, 1982; Sass and Bautz, 1982a,b), 3) the location of RNP granules (e.g. Skaer, 1977; Mott et al., 1980), 4) determinations of total band numbers, band-interband lengths, DNA content or protein coding potentials (reviewed in the Introduction and section 6.3. of the Discussion), and 5) the location of cloned genetic sequences (e.g. Schmidt, 1981; Haenlin et al., 1985; Carmona et al., 1985; Whitmore, 1986; Rykowski et al., 1988).

<D> Investigations of the cytology of the structures formed by transposed DNA segments of mobile elements inserted in the *Drosophila* genome by P element-mediated transformation (Semeshin et al., 1986; Semeshin et al., 1989).

The results of investigations cited above can be taken, often to the contrary of each other, as indicating the possibility of transcription in the interbands, puffs, diffused bands, and bands. Some of those investigations, along with numerous others, have led to two basic hypotheses concerning the significance of the banding pattern, the so-called "genetic hypothesis" and the "epigenetic hypothesis". Ashburner (1980), summarizes the differences as following: in the genetic hypothesis "the banding pattern is determined solely by the nucleotide sequence...the nucleotide sequence may contain "motifs" that determine the association of proteins with the DNA and, hence, the pattern into which the DNA must pack. Thus, band/interband boundaries are predetermined by the nucleotide sequence." Whereas, in the epigenetic hypothesis, "the banding pattern, though reflecting differences in the DNA packing ratio, is a consequence of particular physiological conditions, e.g. the pattern of transcription."

In this respect, the integrated approach taken in this thesis indicates conclusively on the electron microscopic level that:

1) The interband lengths of polytene chromosomes are, on the average, long enough to contain coding sequences as determined by the chromosome mapping of entire Drosophila hydei chromosomes (Results section 5.3.).

2) Although there can be a band by band homology (or structure by structure in the case of tissue specific puffs) between polytene chromosomes of different tissues, some structures display what appears to be band splitting "band modulation" as shown in the comparative mapping of the salivary gland and Malpighian tubule polytene chromosomes of Chironomus th. piger (Results section 5.4.).

3) By the physical manifestation of transcriptionally active regions in the form of visible puffs, there appears to be a correlation not only between the appearance of a puffed region and the transcriptional activity of that region, but also to the level of polyteny in the tissue itself as indicated by the results of the comparison between the puffing patterns of the salivary gland and Malpighian tubule polytene chromosomes of Chironomus th. piger (Results section 5.4.).

4) Even so-called "housekeeping" genes appear to occupy both interband and band areas as demonstrated by the localization of the haemoglobin IV gene on Ch. th. thummi chromosome III (Results section 5.5.3.). This also seems to be the case for developmental genes as determined by Rykowski et al. (1988), light microscopically, for the Notch gene by D. melanogaster.

Unfortunately, the flaired nature of the telomeric region of the third chromosome of Ch. th. thummi made it difficult to make a more precise determination of the extent of the labeling in either the band or interband region. The novel routine chromosome transferal method of in situ hybridized SSP chromosomes from glass microscope slides onto grids for electron microscopic viewing introduced in section 4.3.5. of Methods should definitely aid in further investigations of this aspect of chromosomal research when "housekeeping" genes become available which are in a more suitable position for study.

6.3.4. The relationship between chromosome structure and in situ hybridized DNA probes on SSP chromosomes

The theoretical resolution of autoradiographic detection of radioactively labeled probes has been discussed in detail in a number of sources (e.g. Fischer and Werner, 1971; Rogers, 1969). It should suffice here to note that in comparison to immunogold detection of biotin-labeled probes it has, among others, the following disadvantages: low sensitivity (particularly at the EM level), long autoradiographic exposure times, variability in the results, poor preservation of morphological details, and the relatively large size of the silver grains (ca. 0.2 - 0.4 μm). Even in the case of SSP chromosomes of Drosophila hydei with average interband lengths of 0.33 - 0.38 μm and band lengths of 0.26 - 0.29 μm as determined for the chromosomes X and 4, this means that an average fine silver grain is as large as or larger than these structures. In the case of squash preparations the situation is even worse. Here, based on electron micrographs of a portion of the salivary gland X chromosome of Drosophila melanogaster using the squash and thin sectioning technique, average band and interband thicknesses were calculated of 0.12 μm (reviewed in Beer-mann, 1972). Actually, "the genetic and cytogenetic localization of genes of developmental importance can usually be narrowed down to ten bands or so" (John and Miklos, 1988).

Concerning the immunogold labeling of biotin-labeled DNA on SSP chromosomes, it was shown at the EM level (Results section 5.5.1.) that at the labeled sites the DNP fibers appear to be individual DNA double helixes in completely extended form (i.e. at a ratio of ca. 0.34 kb/ μ m). If one considers first just the immunogold marker, then the resolution is directly proportional to the size of the gold sphere. For example, a 25 nm gold sphere would have a resolution of 73.5 base pairs. Smaller immunogold particles are now commercially available down to sizes of 10, 5, or 1 nm which would increase the resolution even more. One must also take into consideration, however, the length of antibodies involved. This is a difficult question to answer due to the complete lack of actual data concerning the size of antibody/antigen formations. In addition, it has not been determined how far the primary antibodies (rabbit anti-biotin IgG) extend into the DNA structure at antigen (biotin) labeled sites or how far the secondary antibodies (goat anti-rabbit IgG) extend from the colloidal gold particles. For now, one can assume as maximal length those determined for human IgG fragments which are 6.0 - 9.8 nm for the Fab and 4.5 - 9.9 nm for the Fc fragment depending on whether electron microscopy, X-ray diffraction or low-angle X-ray scattering was used (reviewed by Gally, 1973). Taking into consideration the length of two antibodies and a 25 nm gold particle, this would yield as a maximum distance from the target site (the biotin-labeled nucleotide) 46 - 64.4 nm or 135 - 189 base pairs. This is ca. 1/8 - 1/11 the length of an average processed transcript of 1.5 Kb (see Table 13) and still ca. 1/3.4 - 1/4.8 less than one of the shortest processed transcripts, that for ribosomal protein at 0.65 kb.

In the immunogold labeling of biotin-labeled DNA probes on SSP chromosomes at the EM level it is thus apparent that one is no longer dealing with a resolution in the sense of groups of bands and interbands, as in autoradiography or immunofluorescence, but rather in terms of the number of

base pairs covered by the immunogold marker and the antibody/antigene complex.

6.4. Future perspectives

With the combination of the methodology used and introduced in this thesis, the opportunity is now available for the first time to undertake the mapping of genes on polytene chromosomes with ultrastructural resolution on a routine basis.

It would be of particular interest to use this methodology together with the technique of chromosome walking (Bender et al., 1983b). Chromosome walking can be used to investigate at the chromosomal level the genetical organization of genes and gene families which still include those transcriptional units not represented in the processed transcripts such as leaders, trailers, introns, and the flanking landscapes. This technique has been used to study the molecular genetics of, for example, the Bithorax Complex (Bender et al., 1983a, b; Karch et al., 1985) and the rosy and Ace loci (Bender et al., 1983b; Bossy et al., 1984; Hall et al., 1984; Gausz et al., 1986). All of these investigations have been carried out at the LM level to which Bossy (op. cit.) notes "the resolution of the mapping of chromomeric units by in situ hybridization of cloned fragments of the walk is not sufficient to establish direct correlations with transcripts..." The implementation of the immunoelectron methodology based on the SSP chromosome technique should definitely provide in such cases and in other molecular cytogenetical investigations, an important research tool in the future.

APPENDIX

Table 13. Examples of cloned genes in D. melanogaster and the lengths of their transcripts for comparative purposes to theoretical coding potentials determined from measurements of interband lengths in Drosophila

Gene	Length of transcript (kb)
Alcohol dehydrogenase	
Adh	1.3
Actin proteins	
A-1	1.7
A-2	3.3
A-3	1.6
A-4	2.2
A-5	1.6
A-6	1.6
Antennapedia locus	>100
Bithorax locus	>70
Chorion proteins	
S15-1	0.67
S16-1	0.75
S18-1	0.82
S19-1	0.74
S36-1	1.1
S38-1	1.5
Collagen protein	6.4
Glue proteins	
sgs-3	1.1
sgs-4	0.9
sgs-7	0.32
sgs-8	0.36
H, D, L genes (3 genes)	1.7 per gene
Heat shock proteins	
hsp 22	1.03
hsp 23	0.96
hsp 26	1.05
hsp 28	1.25
hsp 68	2.1
hsp 70	2.25
hsp 80	3.7
Larval serum proteins	
LSP-1 α	2.85
LSP-1 β	2.85
LSP-1 γ	2.85
Mobile dispersed	
copia	5
297	6.5
412	7
mdg 1	7.2
mdg 3	5.5
gypsy	7.3
B104	8.5

Myosin heavy chain	19
Ribosomal protein	0.65
Rudimentary locus	7.03
Tubulin proteins	
α -1	1.8
α -2	1.65, 1.8
α -3	1.8, 2
α -4	1.5, 1.7, 1.9, 1.95
Tropomyosin I, II	1.5, 1.8, 2.8
Vitellogenin proteins	
YP-1	1.6
YP-2	1.6
YP-3	1.5

After John and Miklos, 1988, including data from Bautch et al., 1982; Crowley and Meyerowitz, 1984; Griffin-Shea et al., 1982; Hogness et al., 1985; Hovemann and Galler, 1982; Rubin, 1983; Segraves et al., 1983; Spradling and Rubin, 1981; Synder and Davidson, 1983; Meyerowitz and Hogness, 1982; North, 1984.

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